

Insect Clocks

Third Edition



D.S. Saunders

With

C.G.H. Steel, X. Vafopoulou & R.D. Lewis

ELSEVIER

INSECT CLOCKS

Third Edition

This Page Intentionally Left Blank

INSECT CLOCKS

Third Edition

D.S. SAUNDERS

University of Edinburgh

With

C.G.H. STEEL, X. VAFOPOULOU, York University, Toronto, Canada

R.D. LEWIS, University of Auckland, New Zealand

2002



ELSEVIER

Amsterdam • Boston • London • New York • Oxford • Paris • San Diego
San Francisco • Singapore • Sydney • Tokyo

ELSEVIER SCIENCE B.V.
Sara Burgerhartstraat 25
P.O. Box 211, 1000 AE Amsterdam, The Netherlands

© 2002 Elsevier Science B.V. All rights reserved.

This work is protected under copyright by Elsevier Science, and the following terms and conditions apply to its use:

Photocopying

Single photocopies of single chapters may be made for personal use as allowed by national copyright laws. Permission of the Publisher and payment of a fee is required for all other photocopying, including multiple or systematic copying, copying for advertising or promotional purposes, resale, and all forms of document delivery. Special rates are available for educational institutions that wish to make photocopies for non-profit educational classroom use.

Permissions may be sought directly from Elsevier Science via their homepage (<http://www.elsevier.com>) by selecting 'Customer support' and then 'Permissions'. Alternatively you can send an e-mail to: permissions@elsevier.co.uk, or fax to: (+44) 1865 853333.

In the USA, users may clear permissions and make payments through the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA; phone: (+1) (978) 7508400, fax: (+1) (978) 7504744, and in the UK through the Copyright Licensing Agency Rapid Clearance Service (CLARCS), 90 Tottenham Court Road, London W1P 0LP, UK; phone: (+44) 207 631 5555; fax: (+44) 207 631 5500. Other countries may have a local reprographic rights agency for payments.

Derivative Works

Tables of contents may be reproduced for internal circulation, but permission of Elsevier Science is required for external resale or distribution of such material.

Permission of the Publisher is required for all other derivative works, including compilations and translations.

Electronic Storage or Usage

Permission of the Publisher is required to store or use electronically any material contained in this work, including any chapter or part of a chapter.

Except as outlined above, no part of this work may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without prior written permission of the Publisher.

Address permissions requests to: Elsevier Science Global Rights Department, at the mail, fax and e-mail addresses noted above.

Notice

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein. Because of rapid advances in the medical sciences, in particular, independent verification of diagnoses and drug dosages should be made.

First edition 2002

Library of Congress Cataloging in Publication Data

A catalog record from the Library of Congress has been applied for.

British Library Cataloguing in Publication Data

A catalogue record from the British Library has been applied for.

ISBN: 0 444 50407 9

© The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).
Printed in The Netherlands.

This book is dedicated to my father
J.M.K. Saunders
who introduced me to the world of insects;
to my wife, Jean, for her continued support and encouragement;
and to my friends, the late Jürgen Aschoff, Erwin Bünning and Colin Pittendrigh,
giants of chronobiology on whose shoulders I have climbed.

This Page Intentionally Left Blank

PREFACE

IN the twenty years or so since the appearance of the second edition of *Insect Clocks*, the field has come of age. In the early 1970s those of us working on chronobiology knew, of course, that we were dealing with a fascinating and very important branch of biological science. As we now enter the 21st century chronobiology, particularly the study of daily or circadian rhythms, has moved centre-stage, seemingly with hardly a week passing without major advances being published in prestigious international journals. The field is continually in the public eye - and being digested and re-digested in reviews, mini-reviews, mega-reviews and even reviews of reviews! Particular and important impetus to the study of circadian rhythms has been the enormous advance in molecular genetics. This was in its infancy in the 1970s when Ron Konopka and his colleagues isolated and provided initial characterisation of the fruit fly's *period* gene, but was followed during the 1980s and 1990s by an exponential rise in our knowledge and understanding of 'central' circadian mechanisms. The purpose of this book's third edition is to present modern developments securely embedded in a background of the broader, and sometimes older, aspects of insect biological rhythms. The genetics and molecular biology of circadian rhythmicity will not be presented in detail, but only to provide an entrée into that now voluminous literature. There is always a real danger that the speed of advance in molecular genetics may lead to a growing ignorance or collective amnesia, especially among younger colleagues, of the roots of the science. This may lead to a continual re-invention of the wheel, or a failure to address important biological phenomena using modern techniques. An over-simplified view may also mask the complexities of 'time measurement' in biological systems and engender a complacency that we 'understand' the problems involved, when the reality is that we are probably only just beginning to understand them.

Significant advances have been made in all aspects of insect clocks. These include the increasing realisation that circadian rhythmicity is basically a cellular phenomenon so that a complex multicellular organism such as an insect is therefore a collection of interacting oscillators. Other advances involve unravelling the complex and often multiple photoreceptive input pathways, the anatomical location and neurobiology of 'central' behavioural pacemakers, and the uncovering of some of the neural and hormonal outputs to these behaviours. Our knowledge of one of the most important functions of the circadian system - that of seasonal photoperiodism - has also advanced but remains at a mainly phenomenological level, largely because of the enormous complexity of that system. Nevertheless, our appreciation of the probably 'causal' role of circadian rhythmicity in photoperiodic time measurement has matured: Alternative photoperiodic models, such as 'hourglass-like' timers, are now best regarded as also circadian-based, although probably incorporating rather heavily damped oscillators.

Advances in insect chronobiology since publication of the second edition have made it increasingly difficult for one author to cover the entire field. Without turning *Insect Clocks* into just another multi-author treatise, with attendant dangers of overlap, gaps and possible differences of opinion, agreement was made with the publishers that certain aspects of the subject should be covered by 'external contributors'. Thus, excellent accounts of the 'wet' physiology of circadian systems (Chapter 5) are given by Colin Steel and Xanthe Vafopoulou (York University, Toronto) and of mathematical modelling of circadian rhythms (Chapter 7) by Robert Lewis (University of Auckland). The remaining thirteen chapters were written by myself. Introduction of external contributors has inevitably introduced *some* overlap between chapters, but such overlap has been kept to a minimum and, anyway, adds authority to certain aspects of the subject. Material and interpretations presented by the three external contributors

are entirely their own responsibility; likewise, views expressed by myself in the remaining thirteen chapters are my own. I am confident that this spread of authorship has introduced no serious differences of opinion: any slight differences that might be detected by the reader are merely healthy signs of a rapidly developing field of research.

Permissions to reproduce figures from various journals were granted by the following publishing houses: Birkhauser Verlag AG, Basel, Fig 11.19; Blackwell Scientific Publications, Figs 2.6, 12.8 and 14.3; The Company of Biologists, Fig. 9.2; Marcel Dekker Inc., Figs 8.1, 8.6; Elsevier Science, Figs 2.7, 2.22, 8.4, 9.2, 9.3, 9.5, 9.6, 10.4, 10.5, 10.18, 11.15, 11.20, 14.1, 14.4, 14.5; Photochemistry & Photobiology, Fig. 2.20; Sage Publications Inc., Figs 2.19, 2.23, 3.49, 6.6, 13.22; Springer-Verlag, 2.13, 2.23, 4.47, 3.48, 12.9, 15.10, 15.11; Swets & Zeitlinger Publishers, Fig. 6.3; and Taylor and Francis Inc., Fig. 2.8.

As with earlier editions of this book, its writing has been greatly assisted by a number of friends and colleagues, both through their writing and through discussion. Many of them have given permission for the reproduction of figures from their papers; these attributions are given in the respective figure legends. In particular, I would like to acknowledge Fred Kippert for wide-ranging discussions on chronobiology, and David Walker, Robert Saunders and Richard Saunders for help with computation.

Edinburgh, June 2002

D. S. SAUNDERS

CONTENTS

Glossary and List of Symbols	xi
1. Introduction: Rhythms and Clocks	1
2. Circadian Rhythms of Activity in Individual Insects	7
3. Circadian Rhythms of Activity in Populations of Insects	43
4. Circadian Rhythms: Genes and the Feedback Loop	103
5. Physiology of Circadian Systems (by C.G.H. Steel & X. Vafopoulou, Department of Biology, York University, Toronto M3J 1P3, Canada)	115
6. The Multioscillator Circadian System	189
7. Quantitative Models for Insect Clocks (by R.D. Lewis, University of Auckland, New Zealand)	213
8. Circadian Rhythms: Photoreceptor and Clock Location	245
9. Photoperiodism and Seasonal Cycles of Development	271
10. The Photoperiodic Response	299
11. Circadian Rhythms in Photoperiodism	339
12. The Photoperiodic Counter	377
13. Photoperiodic Time Measurement: the Clock-Counter Mechanism	395
14. Photoperiodic Photoreceptors and Clock Location	433
15. Other Types of Insect Clocks	449
16. Clock Complexity: The Way Forward?	473
References	485
Index	551

This Page Intentionally Left Blank

GLOSSARY AND LIST OF SYMBOLS

A. Circadian Biology

This terminology is based on the Circadian Vocabulary of Aschoff, Klotter and Wever (1965), with additions.

Activity time (α). Time in a 'sleep-wake' or activity cycle when the animal is active.

Advance phase-shift ($+\Delta\phi$). One or more periods shortened after perturbation by a light or temperature signal causing the overt phase to occur earlier in steady-state than in the control (unperturbed) oscillation.

Aestivation. A summer dormancy (diapause or quiescence). An aestivation diapause is frequently induced by long day length.

Amplitude. The difference between the maximum (or minimum) value and the mean value in a sinusoidal oscillation. In 'population' rhythms it often also refers to the number of individuals emerging or eclosing, etc., through a particular gate.

Aschoff's rule ($\tau_{DD} < \tau_{LL}$, nocturnal; $\tau_{DD} > \tau_{LL}$, diurnal). Period of the free-running oscillation (τ) lengthens on transfer from DD to LL, or with an increase in light intensity, for dark-active animals, but shortens for light-active animals (Pittendrigh, 1960).

Asymmetrical skeleton photoperiod. A skeleton photoperiod comprising a 'main' photoperiod (e.g. 4 to 12 hours) and a short supplementary light pulse which systematically scans the accompanying 'night' (Pittendrigh and Minis, 1964).

Bistability phenomenon. Ability of an endogenous oscillation to adopt either of two distinct phase relationships to a symmetrical skeleton photoperiod (PP_s), depending on (1) the phase-point in the oscillation which is illuminated first, and (2) the value of the first interval. In *Drosophila pseudoobscura* the bistability phenomenon is observed between PP_s 10.3 and PP_s 13.7 (Pittendrigh, 1966).

Bivoltine. An insect life cycle with two generations per year.

Circadian (rhythm). An endogenous oscillation with a natural period (τ) close to, but not necessarily equal to, that of the solar day (24 hours).

Circadian rule. The relationship of the ratio of activity time (α) to rest time (ρ), and the total amount of activity, to the light intensity. In many vertebrate species α/ρ and activity increase with light intensity in day-active animals, but decrease in night-active animals (Aschoff, 1965).

Circadian system. The sum total of circadian oscillators (driving oscillators) and driven rhythms in an organism. (These may be independent, loosely coupled or coupled.)

Circadian time (Ct). Time scale (in hours, radians or degrees) covering one full circadian period of an oscillation. The zero point is defined arbitrarily. In *Drosophila pseudoobscura* the point at which the oscillation enters darkness from LD 12:12 or from LL is considered to be the beginning of the subjective night (Ct 12); the first eclosion peak occurs at Ct 03, 15 hours after the LL/DD transition (Pittendrigh, 1966).

Circadian topography. A three-dimensional plot of diapause incidence against the length of the photoperiod and the period of the driving light cycle (T). In species with a circadian photoperiodic clock the topography shows 'mountains' of diapause at 24-hour intervals.

Circannual. An endogenous oscillation with a natural period (τ) close to, but not equal to, a year. In the beetle *Anthrenus verbasci* (Blake, 1958) τ is 41 to 44 weeks.

Circasyzygic. An endogenous oscillation with a natural period which approximates to the interval between successive spring or neap tides (14.7 days) (= semilunar rhythm).

Cophase (θ). The interval between the end of the light perturbation and the centroid of the eclosion peaks (Winfree, 1970).

Crepuscular. Twilight active.

Critical daylength (or nightlength). The length of the light (or dark) fraction of the light/dark cycle that separates a 'strong' long day lengths from a 'strong' short day length in the photoperiodic response curve, i.e. a 50 per cent response.

Day-neutral (species, response, etc.). Apparently with no reaction to photoperiodic influences.

Delay phase-shift ($-\Delta\phi$). One or more periods lengthened after perturbation by a light or temperature signal causing the overt phase to occur later in steady state than in the control (unperturbed) oscillation.

Diapause. A period of arrest of growth and development which enables the species to overwinter (hibernate) or aestivate, or to synchronise its development cycle to that of the seasons. In most cases diapause involves the cessation of neuroendocrine activity, and is most frequently induced by photoperiod.

Diurnal. Occurring during the day or light period of the cycle, day-active (cf. Nocturnal). An older usage of this term denoting a daily cycle is not used in this book.

Endogenous rhythm (or oscillation). A periodic system which is part of the temporal organisation of the organism. It is self-sustaining, i.e. it 'free-runs' in the absence of temporal cues such as the daily cycle of light and temperature.

Entrainment. The coupling of a self-sustained oscillation to a *Zeitgeber* (or forcing oscillation) so that both have the same frequency ($\tau = T$) (synchronisation) or that the frequencies are integral multiples (frequency demultiplication). Entrainment is possible only within a limited range of frequencies.

Eudiapause. A facultative cessation of development with species-specific sensitive periods and diapausing instars. In favourable conditions development proceeds unchecked; as unfavourable periods approach diapause supervenes. This type of diapause is usually induced by photoperiod and terminated by a period of chilling or by a change in the level of the temperature (Müller, 1970).

Exogenous rhythm. A rhythm of activity which is a direct response to the environmental cycle of light and temperature. In the absence of these variables the rhythm does not persist.

External coincidence. A model for the photoperiodic clock in which light has a dual role: (1) it entrains and hence phase-sets the photoperiodic oscillation, and (2) it controls photoperiodic induction by a temporal coincidence with a photoperiodically-inducible phase (ϕ_i) (Pittendrigh and Minis, 1964).

Fixed point. A point in the circadian oscillation at which a light pulse leaves the oscillation at the same circadian time it was when the pulse started, regardless of the duration of the pulse. In *Drosophila pseudoobscura* the theoretical position of the fixed point is at Ct 10.75 (Johnsson and Karlsson, 1972b).

Forcing oscillation (see also *Zeitgeber*). An oscillation or periodic environmental factor capable of synchronising or entraining another oscillation.

Free-running period (τ). The period of an endogenous oscillator, revealed in the absence of a forcing oscillation or *Zeitgeber* (i.e. in constant temperature and DD, or LL).

Free-running rhythm. A biological rhythm or oscillation in its 'free-running' condition (unentrained).

Frequency. The reciprocal of period.

Frequency demultiplication. The entrainment of an endogenous oscillation to a *Zeitgeber* when the frequencies are integral multiples, e.g. the entrainment of a circadian oscillation ($\tau \sim 24$ hours) to environmental light cycles with $T = 4, 6, 8$ or 12 hours, resulting in an entrained steady state with a period of exactly 24 hours.

Gate. The 'allowed zone' of the cycle, dictated by the circadian clock, through which insects may emerge, hatch, etc. If a particular insect is not at the 'correct' morphogenetic stage to utilise one gate it must wait a full 24 hours for the next (Pittendrigh, 1966).

Hibernation. The state of dormancy (diapause or quiescence) which occurs during the winter months.

Hourglass. A non-repetitive (i.e. non-oscillatory) timer which is set in motion at, say, dawn or dusk and then runs its allotted time. Hourglass-like timing devices may be found in aphid photoperiodism (Lees, 1965) and in certain other clock phenomena. They may be merely heavily damped circadian oscillators.

Intermediate photoperiodic response. The photoperiodic response in some 'univoltine' species in which nondiapause development is restricted to a very narrow range of day lengths (18 to 20 hours); with both longer and shorter day lengths diapause occurs (Danilevskii, 1965).

Internal coincidence. A model for the photoperiodic clock in which two or more oscillators are independently phase-set by dawn and dusk, and photoperiodic induction depends on the phase-angle between the two (Pittendrigh, 1972; Tyshchenko, 1966).

Interval timer. (a) A non-repetitive timer or hourglass (Lees, 1965). (b) A type of oscillatory clock which dictates the times of the day at which a particular event (e.g. eclosion) can occur - in contrast to 'pure rhythms' or 'continuously consulted' clocks (Pittendrigh, 1958).

Isoinduction surface, see Circadian topography.

Long-day (species, response, etc.). The photoperiodic response in which the insects grow and develop during the

- summer months at long day length, but enter diapause in the autumn as the days shorten.
- Long-day-short-day response.** The photoperiodic response which requires short days following long days for its operation.
- Multivoltine.** An insect life cycle with many generations per year.
- Night-interruption experiment.** An experiment in which an insect's photoperiodic response is investigated by asymmetric skeleton photoperiods.
- Nocturnal.** Occurring during the night or dark period of the cycle, night-active (cf. Diurnal).
- Oligopause.** A facultative arrest of development often with the induction and termination of diapause under photoperiodic control (Müller, 1970).
- Oscillator.** (A-oscillator). In Pittendrigh's terminology (1960, 1967) the A-oscillator is the self-sustained and light-sensitive oscillator whose period is temperature-compensated and which drives the temperature-sensitive B-oscillator (rhythm) which more immediately controls the overt rhythm of activity (e.g. eclosion). The circadian pacemaker.
- Parapause.** An obligatory diapause observed in univoltine species. Clearly defined inductive periods and diapause supervene in every generation in a species-specific instar. Onset appears to be independent of the environment (Müller, 1970).
- Period.** The time after which a definite phase of the oscillation recurs. In biological systems it should be stated what overt phase reference point (ϕ_0) has been used to determine the period, e.g. onset of activity, or median of eclosion peak, etc.
- Phase.** Instantaneous state of an oscillation within a period.
- Phase-angle (ψ).** Value on the abscissa corresponding to a point of the curve (phase) given either in radians, in degrees, or in other fractions of the whole period. It can be given in time units if the length of the period is stated.
- Phase angle difference.** Difference between two corresponding phase angles in two coupled oscillators, given either in degrees of angle or in time units.
- Phase response curve (PRC).** Plot of phase shift ($\Delta\phi$) (magnitude and sign) caused by a single perturbation at different phases (circadian times) of an oscillator in free-run.
- Phase shift ($\Delta\phi$).** A single displacement of an oscillation along the time axis may involve either an advance ($+\Delta\phi$) or a delay ($-\Delta\phi$).
- Photonon.** The kinetics of a clock after the onset of the light (Truman, 1971b).
- Photoperiod.** The period of light in the daily cycle (day length) measured in hours. See Photophase.
- Photoperiodic counter.** That aspect of the photoperiodic response which consists of a temperature-compensated mechanism accumulating 'information' from successive photoperiodic cycles.
- Photoperiodically inducible phase (ϕ_i).** A hypothetical phase-point in an oscillator (or perhaps a driven rhythm) which is light-sensitive, and an integral part of the external coincidence model for the photoperiodic clock (Pittendrigh, 1966).
- Photoperiodic response curve (PPRC).** The response of a population of a particular insect to a range of stationary photoperiods (DD to LL) usually including the critical day length.
- Photophase = photoperiod, day length** (Beck, 1968).
- Quiescence.** A state of dormancy directly imposed by adverse factors in the environment (e.g. cold torpor, dehydration) (cf. Diapause).
- Range of entrainment.** Range of frequencies within which a self-sustained oscillation can be entrained by a *Zeitgeber*. For most organisms the range is from about 18 to 30 hours.
- Rest time (ρ).** Time in a 'sleep-wake' cycle in which the organism is inactive (Aschoff, 1965).
- Rhythm.** A periodically recurring event. In Pittendrigh's (1967) sense the word is restricted to the driven elements (i.e. the temperature-dependent B-oscillations) directly coupled to the light sensitive driver (A-oscillation).
- Required day number (RDN).** The temperature-compensated number of inductive photoperiods in the photoperiodic counter, required to raise the incidence of diapause in a particular day's batch to 50 per cent. Equivalent to the 'critical day number' of Tyshchenko et al. (1972).
- Resonance experiment (= T-experiment).** An experimental design in which the photophase is held constant

- but the period of the driving light cycle (T) varied (e.g. from 12 to 72h).
- Scototonon.** The kinetics of a clock after the onset of the dark (Truman, 1971b).
- Scotophase.** The dark period, or nighttime, of the diel-cycle (Beck, 1968).
- Semivoltine.** An insect life cycle which occupies a 2-year period, i.e. half a generation per year.
- Sensitive period (SP).** The period of an insect's life-cycle when it is sensitive to photoperiodic control of diapause induction or termination.
- Short-day.** (Species, response, etc.) The photoperiodic response in which the insects grow and develop at short day length, but enter diapause (aestivation) during the summer months when days lengthen.
- Short-day-long-day response.** A photoperiodic response which requires long days following short days for its operation.
- Skeleton photoperiod.** A light regime using two shorter periods of light to simulate dawn and dusk effects of a longer, complete, photoperiod (PP_c). See Symmetrical and Asymmetrical skeleton.
- Singularity (T*S*).** A critical annihilating light pulse of a particular duration (S*) or intensity placed a certain time (T*) after the LL/DD transition (i.e. at a particular circadian time) which puts the clock in a non-oscillatory state (i.e. stops the clock). In *Drosophila pseudoobscura* T*S* is a 50-second pulse of dim blue light (10 $\mu\text{W}/\text{cm}^2$) placed 6.8 hours after the LL/DD transition (i.e. at about Ct 18-19) (Winfree, 1970).
- Subjective day.** The first half of the circadian cycle (Ct 0 to 12) of an oscillation, and that half in which 'day' normally occurs.
- Subjective night.** The second half of the circadian cycle (Ct 12 to 24) of an oscillation, and that half in which 'night' normally occurs.
- Symmetrical skeleton photoperiod (PP_s).** A skeleton photoperiod comprised of two short pulses of equal duration which may simulate a complete photoperiod (PP_c) (Pittendrigh and Minis, 1964).
- Synchronisation.** State in which two or more oscillations have the same frequency due to mutual or unilateral influences. See also Entrainment.
- T-experiment,** see Resonance experiment.
- Thermoperiod.** A daily temperature cycle which may be sinusoidal, 'square-wave', etc., and may act as a *Zeitgeber*.
- Token stimulus.** A seasonal signal which serves to indicate the approach of adverse conditions (e.g. winter) but is itself not adverse (e.g. short photoperiod) (Lees, 1955).
- Transformation curve.** A plot of the circadian time of an oscillation at the end of a light-pulse as a function of the circadian time at the beginning of the pulse (Johnsson and Karlsson, 1972b).
- Transients.** One or more temporary oscillatory states between two steady states caused, for instance, by light or temperature perturbations.
- Univoltine.** An insect life cycle with one generation per year.
- Voltinism.** Referring to the number of generations per year.
- Zeitgeber.** That forcing oscillation which entrains a biological oscillation, e.g. the environmental cycle of light and temperature.
- Zeitgeber time (Zt).** Time of environmental cycle measured in hours, usually, in the, case of light, after the lights-on signal or 'dawn'.
- Zeitgedächtnis.** The 'time-memory' of bees.
- Zeitnehmer.** ('time taker'). An input pathway that is itself rhythmically regulated by feedback from an oscillator. It will therefore create a rhythmic input to the 'central' oscillator even in constant environmental conditions.
- Zeitsinn.** The 'time-sense' (= time memory) of bees.

Symbols

- L** Light fraction of the cycle (intensity may be specified).
- D** Dark fraction of the cycle.
- LD** Light/dark cycle. LD 4: 20 represents 4 hours of light and 20 hours of darkness in each 24-hour cycle.
- LL** Continuous light.
- DD** Continuous darkness

τ	Natural period of a biological oscillation as revealed in 'free-running' conditions.
T	Period of <i>Zeitgeber</i> .
ϕ	Phase point.
ϕ_r	Phase reference point. ϕ_r for environmental light cycle may be beginning of light-pulse; ϕ_r for the oscillator in <i>Drosophila pseudoobscura</i> is the easily assayed point in the phase response curve where a 360° phase-jump occurs (Ct 18.5); ϕ_r for the rhythm (in <i>D. pseudoobscura</i>) is the median of pupal eclosion. In other systems it may be, for instance, the onset of locomotor activity.
ϕ_i	The photoperiodically inducible phase.
ψ	Phase relationship
ψ_{RL}	Phase angle difference between phase reference point of rhythm and light cycle
ψ_{RO}	Phase angle difference between phase reference point of rhythm and oscillation
ψ_{OL}	Phase angle difference between phase reference point of oscillation and light
$\Delta\phi$	Phase shift.
$+\Delta\phi$	Advance phase shift.
$-\Delta\phi$	Delay phase shift.
α	Activity time.
ρ	Rest time.
θ	Cophase. The time interval between the end of the light perturbation and the centroid of the eclosion peaks.
Ct	Circadian time.
Zt	<i>Zeitgeber</i> time.

This Page Intentionally Left Blank

CHAPTER 1

INTRODUCTION: RHYTHMS AND CLOCKS

While the earth remaineth, seedtime and harvest, and cold and heat, and summer and winter, and day and night shall not cease. Genesis 8:22

EVER since life first appeared on this planet it has been subjected to daily cycles of light and dark, and to seasonal cycles of climatic change, caused by the rotation of the earth around its axis and around the sun. Marine and inter-tidal organisms have in addition been subjected to tidal and lunar periodicities. Only those animals that invaded the depths of the ocean, or underground caves and rivers, have avoided this fluctuating environment. Other species - especially those on the land, where daily and seasonal changes may include violent fluctuations in temperature and humidity - have developed strategies to counteract or to exploit this periodicity. The majority of insects, for example, show daily and annual cycles of activity and development. They may be nocturnal, diurnal or crepuscular. They may hibernate or aestivate. Plants may produce leaves or flowers only at certain seasons, and flowers may open and close at particular times of the day.

Some of these phenomena are direct responses to environmental changes, but many more are overt manifestations of an endogenous periodicity. These innate rhythms must have astounded early workers such as the French astronomer De Mairan who discovered (in 1729) that the daily leaf movements of *Mimosa* would persist in constant darkness. The oscillations underlying such phenomena are now known to provide a temporal organisation for physiological and behavioural activities in practically every group of organisms, including prokaryotes such as some blue-green 'algae' (see Johnson et al. 1998). Of particular interest are those endogenous oscillations that evolved with a periodicity close to 24 hours (circadian rhythms). These are used by animals and plants to 'time' daily events and thus allow the organism to perform functions at the 'right time of the day', or to attain synchrony with other individuals of the population. It is clear that these circadian oscillations in the cell and the organism have evolved to match almost exactly the oscillations in the physical environment. In the fruit fly *Drosophila melanogaster* the circadian period is inherited and important advances have been made in understanding the genetics and molecular biology of these rhythms (Chapter 4). These rhythms, therefore, are not 'imposed' on the organism by the environment, neither are they 'learned'; they are part of the genome and a product of evolution. The natural cycles of light and temperature, however, do serve to *entrain* and phase-control these endogenous oscillators so that under natural conditions their periods become exactly 24 hours and the overt events they control achieve a particular phase relationship to the environmental periodicity. In the absence of temporal cues from the environment (i.e. in darkness and constant temperature) the rhythms 'free-run' and reveal their own natural period (τ) which is close to, but significantly different from that of the solar day. The observation that this period is temperature-compensated, and that the rhythms are used by the organisms to measure the passage of time (Pittendrigh, 1954, 1960), justifies the use of the term 'biological clock'.

Apart from circadian rhythms which have evolved as a match to the 24-hour periodicity

of the earth's rotation around its axis, endogenous oscillations with tidal (~12.4 hours), semilunar (~14.7 days), lunar (~29.4 days) or annual (~a year) periods are also to be found in organisms, including the insects. In many cases the endogenous nature of these rhythms has been demonstrated by allowing them to 'free-run' in the absence of the environmental cues (*Zeitgeber*) which normally entrain them.

The brief account of these biological oscillations given above - and the more extensive description of their properties given later in this book - amply demonstrate their endogenous nature. They are, in fact, every bit a part of the organism as its morphological organisation. Some investigators, however - principally Brown (1960, 1965) - at one time held an alternative view, namely that all of the observed periodicities were in some way exogenously controlled by "subtle geophysical forces" associated with the solar day. These 'forces' were thought to include air pressure, periodic fluctuations in gravity associated with the earth's rotation in relation to the sun and the moon, or cosmic ray intensity - which remained unaccounted for in laboratory experiments in which the obvious periodicities (light, temperature, etc.) had been eliminated. This view will receive no further attention in this book. As a partial answer to the endogenous-exogenous controversy, Hamner et al. (1962) maintained a number of organisms at the South Pole on a turntable arranged to rotate once every 24 hours counter to the earth's own rotation, thereby eliminating most diurnal variables. Under these conditions several rhythmic systems, including the pupal eclosion rhythm in *Drosophila pseudoobscura* (Chapter 3), continued to show a circadian periodicity apparently unaffected by either their location at the South Pole or by their rotation on the turntable. Therefore, as far as these experiments or their results allow, the data support the endogenous hypothesis.

Using the clock analogy for these biological rhythms there is an interesting parallel between the development of man-made 'time-pieces' and those 'clocks' found in nature. Early man was aware of the passage of time by watching the movement of the sun, moon, and stars, or by observing the movement of the sun's shadow on the ground or on a dial. Such methods, of course, have nothing to do with clocks. Neither have the *direct* responses of animals and plants to daily periodicities. These exogenous effects are widespread in nature and in some animals the observed rhythm of activity appears to be related, at least in part, to the immediate effects of the daily changes in light intensity. Under field conditions most daily rhythms - although innate - are nearly always strongly modulated by the immediate character of the environment, particularly the rapid changes in light intensity at dawn and dusk. These 'masking' effects will be discussed only where they modify an endogenous periodicity.

The first man-made time-measuring devices were probably sand-glasses, clepsydras (water clocks) and candles. These 'clocks' did not oscillate and had to be reset or 'turned over' once all the water or sand had run out, or the candle burnt to the bottom. This type of device finds its equivalent in some of the 'hourglass'-like timers thought to perform night-length measurement in some insects which, after measuring the duration of the dark period, require to be 'turned over' by light before they can function again (see Chapter 11).

Mechanical clocks introduced in the fourteenth and fifteenth centuries were either weight-driven or spring-driven and incorporated oscillatory devices that ran continuously so long as the weight was raised or the spring wound up. These find their counterpart in the free-running biological oscillations mentioned above. The escapement in these early clocks consisted of a crown wheel and a verge and foliot. The system was not isochronous and the clocks so constructed tended to lose or gain up to 15 minutes every day. In the seventeenth century the incorporation of a pendulum with an escapement to maintain constant amplitude introduced isochrony to the clock, and brought the error down to about 10 seconds per day.

This pendulum analogy and sine-wave representation of the oscillation's time course remain instructive and useful in model building.

For really accurate time measurement temperature-compensation is required. In man-made clocks an uncompensated pendulum lengthens as the temperature rises and therefore swings more slowly. By the eighteenth century George Graham had compensated for temperature changes by using a mercury-vial pendulum. When the quantity of mercury was correctly adjusted its thermal expansion raised the centre of gravity to compensate for the lengthening of the pendulum rod. Graham's clock varied by as little as one second per day; Harrison's grid-iron pendulum, which operated on a similar principle, later cut this error down to less than one second. In biological systems most physiological processes more than double their rate with every 10°C rise in temperature, and such temperature effects would render time measurement impossible. However, during evolution this challenge has been met: most biological oscillators with a 'clock' function have a temperature co-efficient (Q_{10}) between 0.85 and 1.1. This property is an absolute functional prerequisite for a clock mechanism. It is also essential for effective entrainment by a natural (24 hour) *Zeitgeber* because if the oscillator had a Q_{10} of 2.0 or more it would, at some temperatures, fall outside the limits within which the light-cycle could hold it. The manner in which temperature-compensation is achieved in biological clocks, however, remains obscure (see Chapters 4 and 16).

Defining properties of a biological clock are therefore as follows. (1) They show an overt rhythm, driven by oscillators that persist ('free-run') in the absence of environmental signals (thereby attesting to their endogeneity). (2) The free-running rhythm (period τ) is close, but rarely equal to, the environmental periodicity it has evolved to match. (3) The endogenous period shows a well-defined homeostasis when exposed to a wide range of physical, chemical and biological variables, most notably temperature (i.e. the period is temperature compensated). And (4) the ability to synchronise (entrain) to environmental variables so that τ becomes equal to the period of the entraining agent (*Zeitgeber*), and adopts an adaptive phase relationship to it. These properties are all shown by circadian, circatidal, circa-semilunar, circalunar and circannual rhythms. In addition, some ultradian (short period) rhythms, whilst lacking properties (2) and (4), are nevertheless free-running and temperature compensated. For these reasons they may operate as biological clocks providing temporal organisation within the cell or for some behavioural functions.

In man-made clocks hourglasses clearly antedate oscillators, but the reverse may be the case in biological systems. Pittendrigh (1966) suspected that circadian oscillations - which occur in all eukaryotes (and some prokaryotes) - possessing the common but seemingly 'improbable' properties of accuracy and temperature-compensation are monophyletic in origin and therefore very ancient. Although their original functional significance is unclear, they are now widely used for the purposes of chronometry. In many species of animals and plants the circadian system may also be causally involved in the measurement of day- or night-length, or 'classical' photoperiodism. In some insects, on the other hand, this function may be performed by means of an 'hourglass' rather than by circadian oscillations - which they surely must possess. Evidence for an evolutionary convergence such as this suggests that the adoption of hourglass-like timers for photoperiodic time measurement is a comparatively recent event. It is suggested here (see Chapter 11) that hourglass-like timers may be heavily damped circadian oscillators.

Many aspects of insect physiology and behaviour are clock-controlled. There are, for example, daily rhythms of general locomotion, feeding, mating, oviposition, pupation, and pupal eclosion, in which these activities are restricted to a particular part of the day or night. Photoperiodism also involves a clock measuring day- or night-length, the most frequent

response being the seasonal appearance of a dormant stage (diapause) in the life cycle. The adaptive significance of diapause is clear, but it is not always easy to see the adaptive significance of daily rhythms, and in the absence of concrete experimental evidence most conclusions must remain conjectural. However, adults of *Drosophila pseudoobscura* emerge from their puparia close to dawn when the relative humidity of the air is at its height, and it is known that success in the act of eclosion is greatest under these conditions (Pittendrigh, 1958). Cycles of feeding may be correlated with the supply of food: the classical example of this is probably the 'time-memory' (*Zeitgedächtnis*) of bees. Bees can be 'trained' to visit a food source at a particular time of the day (Beling, 1929), this mechanism ensuring that they visit nectar sources every day at the same time. The significance of this behaviour lies in the observation that not only do flowers open and close at particular hours, but that nectar production is also a circadian event (Kleber, 1935). In many cases the selective advantage of an event being clock-controlled lies in the synchrony attained between individuals of the population. Mating rhythms of certain Diptera, for example, ensure that all sexually active individuals in the population are looking for mates at the same time and thereby increase the likelihood of successful encounters between the sexes. Conversely, differences in mating times between closely related species are known to provide effective mechanisms for genetic isolation (Tychsen and Fletcher, 1971).

Biological clocks have been classified in a number of ways. Pittendrigh (1958) differentiated (1) 'pure' rhythms, such as colour change in the crab *Uca pugnax* (Brown et al., 1953), from (2) 'interval timers', in which a particular event such as pupal eclosion occurs at a particular time of the day, and (3) 'continuously consulted' clocks such as the bees' *Zeitgedächtnis* and time-compensated sun orientation in which time may be 'recognised' at any time of the day. Lees (1960a) used the term interval timer to describe some of the non-oscillatory hourglass-like timing devices in aphids. In a later paper Truman (1971d) proposed that animal clocks fall into two well-defined groups. In Type I, such as the rhythm of pupal eclosion in *Drosophila* spp. (Pittendrigh, 1966) and *Antheraea pernyi* (Truman, 1971a), the compound eyes (or other 'organised' photoreceptors) are not essential for entrainment, the relevant photoreceptors lying in the brain itself. These rhythms also damp out in continuous light of moderate intensity, and the magnitude of the phase-shifts generated by quite short light perturbations may be in the order of 10 hours or more (for *D. pseudoobscura*). These clocks are generally associated with developmental rhythms such as hatching, moulting, eclosion or release of brain hormones. Truman also placed photoperiodism in this category. In Type II clocks, such as those controlling locomotor activity rhythms, the compound eyes are the principal and sometimes the only photoreceptors involved and the brain itself may be insensitive to light. These rhythms 'free-run' in both DD and LL of quite high intensity and the phase-shifts generated by light perturbations are usually much smaller than in Type I. Truman included *Zeitgedächtnis* and time-compensated sun orientation in this category solely because of their association with locomotor activity. The distinction between developmental rhythms which can only be appreciated in mixed-age populations, and those such as general locomotor activity which are performed repeatedly by individual insects often over quite long periods of time, is certainly a useful one. Consequently this distinction is used in the present book, and forms the basis for the first two chapters.

ANNOTATED SUMMARY

1. Insects, like other organisms, evolved in an environment dominated by daily, monthly, annual and, in some cases, tidal periodicities.

2. These environmental periodicities are frequently matched by an appropriate *endogenous* rhythmicity that is a constituent and characteristic physiological feature of living tissue. Insects may thus display circadian (~24 hours), circa-tidal (~12.4 hours), semi-lunar or circasyzygic (~14.7 days), circa-lunar (~29.4 days) or circannual (~a year) rhythms.
3. Defining clock-like properties of biological 'circa-rhythms' are thus endogeneity, a period (τ) close to the environmental cycle, accuracy, temperature-compensation, and an ability to entrain to the appropriate environmental variable or *Zeitgeber*, thereby attaining a functional phase relationship between internal physiology and the world outside. They provide insects with a 'temporal organisation' allowing them to perform functions with a selective advantage at the 'right time of the day', or to 'measure time' as 'biological clocks'.
4. Biological clocks control a wide variety of behavioural and physiological activities in insects. These include daily rhythms of locomotion, feeding, mating, oviposition, pupation and eclosion. These rhythms may operate either in individual insects or in populations. Clocks also control cuticle deposition, metabolism and the seasonal control of alternate developmental pathways (photoperiodism).

This Page Intentionally Left Blank

CHAPTER 2

CIRCADIAN RHYTHMS OF ACTIVITY IN INDIVIDUAL INSECTS

All things from eternity are of like forms and come round in a circle.
 Marcus Aurelius Antoninus.

CONTENTS

Introduction	7
A. <i>Activity in Light/Dark Cycles</i>	8
B. <i>The Endogenous Nature of Activity Rhythms</i>	10
1. 'Free-running' behaviour in the absence of temporal cues	10
2. Insects living in habitats with little or no environmental periodicity	13
3. The effects of constant light (LL)	14
(a) 'Aschoff's rule' and the 'circadian rule'	14
(b) Does constant light (LL) 'stop' the clock?	16
4. Homeostasis of the natural circadian period (τ)	17
(a) Temperature compensation	17
(b) The effects of D ₂ O and lithium	20
5. Bimodal activity patterns	21
6. Circa-bi-dian rhythms (day-skipping)	25
C. <i>Developmental plasticity of the circadian period</i>	25
D. <i>Exogenous and 'Masking' Effects</i>	27
E. <i>Entrainment by Light and Temperature</i>	28
1. Entrainment by light cycles	28
2. Phase-shifting by light pulses, and the phase-response curve (PRC)	30
3. Spectral sensitivity of photic entrainment	35
4. Entrainment by temperature cycles	35
5. Entrainment by other non-photic <i>Zeitgeber</i>	39
F. <i>Clock 'complexity'</i>	41
Annotated Summary	41

INTRODUCTION

INSECTS, like other organisms, usually restrict their activity to certain times of the daily cycle. In natural conditions, or in the artificial light and temperature cycles provided in the laboratory, they may be - with respect to a particular activity - either night-active (nocturnal), day-active (diurnal) or twilight-active (crepuscular). The mechanisms controlling these activity rhythms

may be exogenous (i.e. a direct response to environmental changes) or endogenous (i.e. controlled by an underlying circadian oscillation, or oscillations, which are a part of the physiological make-up of the organism). Most activity rhythms have proved to be a 'mixture' of endogenous and exogenous components. Overt rhythm of activity, although controlled by an endogenous oscillation, are continuously modulated by the direct effects of the environmental cycles of light and temperature, particularly the abrupt changes in light intensity at dawn and dusk. Here we are mainly interested in the endogenous aspects of rhythmic phenomena because the intrinsic and self-sustained physiological oscillations controlling them function as 'biological clocks', and provide temporal organisation for a wide array of behavioural activities.

This chapter is concerned with those aspects of activity such as general locomotion, flight, feeding, and oviposition which are performed repeatedly by individual insects and may persist as a daily rhythm for quite long periods of time. 'Once-in-a-lifetime' events such as egg hatching, moulting, pupation and adult eclosion - which may also be governed by an on-going circadian oscillation - will be discussed in Chapter 3: questions of photoreception and the location of the 'clock' will be dealt with in Chapter 8. Activity rhythms have been studied in a wide range of insect types. Here we will illustrate the general properties of the circadian organisation of these rhythms using some of the most intensively investigated systems. These include: general locomotor activity of cockroaches (Harker, 1956; Roberts, 1960), crickets (Lutz, 1932; Nowosielski and Patton, 1963), beetles (Lohmann, 1964; Birukow, 1964) and stick insects (Eidmann, 1956; Godden, 1973); and flight activity in mosquitoes (Jones et al., 1967; Taylor and Jones, 1969) and other species of Diptera (Roberts, 1956; Brady, 1972). Emphasis is placed on more recent papers or on more fully investigated species.

A. ACTIVITY IN LIGHT/DARK CYCLES

The entomological literature contains hundreds of papers concerned with the daily activity patterns of insects in field conditions. Few of these will be considered in this book because the endogeneity of the majority of these rhythms remains to be established. One example, however, is the massive work of Lewis and Taylor (1965) describing the flight activity rhythms of about 400 species in thirteen insect orders, recorded by suction trapping in a number of sites, mainly in England. This study showed that different species fly at different times of the day or night, most of them being unimodal, but some bimodal. Night flight is relatively rare in the Coleoptera, Megaloptera, Hymenoptera, Strepsiptera, Cyclorrhapha, Brachycera, Thysanoptera and Hemiptera, but predominates in the Neuroptera, Nematocera, Trichoptera and Lepidoptera. Predators, leaf- and flower-feeders, which tend to be 'visual' insects, are usually diurnal; those that feed on decaying matter or on fungi, on the other hand, and which probably find their food by olfactory means, tend to fly in dim light.

Most laboratory work on insect activity has been with either those species in which rhythms are easily recorded (cockroaches, crickets and larger flies, for example) or those that are important for economic or biological reasons (mosquitoes and fruit flies, for example). Further discussions will be largely restricted to these types.

Cockroaches are almost entirely nocturnal in their habits. Under natural and laboratory conditions activity generally commences at or soon after dusk and continues more or less throughout the dark period; the insects become inactive during the day (Gunn, 1940; Mellanby, 1940; Harker, 1954; Roberts, 1960). In the laboratory their large size makes the recording of their activity a relatively easy task. Harker (1956, 1960a), for example, recorded locomotor activity of *Periplaneta americana* in rocking actographs or in phototransistors using very dim

red light, or by attaching a fine wire to the pronotum which wrote on a smoked drum when the insect moved. Roberts (1960) and a number of other authors have used running wheels. In an artificial cycle of 12 hours light and 12 hours dark (LD 12:12) most cockroaches commence activity shortly after the onset of darkness. Considerable variation between individual insects, and between sex, age and physiological state is apparent, however. Roberts (1960) used males of *Leucophaea maderae*, *Byrsotria fumigata* and *P. americana* in preference to females because their activity was 'less erratic'. Leuthold (1966) found that the activity rhythm of female *L. maderae* varied with the insect's reproductive state, locomotion being suppressed when mature eggs were present in the lower reproductive tract. Working with mature adult females of *P. americana*, Lipton and Sutherland (1970) found no activity rhythm that was obviously related to the lighting regime, and similarly concluded that the reproductive cycle interfered with the normal expression of the rhythm. Virgin females, on the other hand, exhibited an entrained rhythm very similar to that shown by adult males. Amongst the males they also found considerable variability. The majority showed a clearly entrained rhythm of activity, or at least a weak nocturnal rhythm or 'pattern', but about 4 per cent were apparently random in their activity. Most of those with a well-marked rhythm showed the typical onset of activity within the first few hours of dark, but over 30 per cent showed a secondary active phase in the first few hours of light. A similar variation in rhythmicity has been recorded by Nishiitsutsuji-Uwo et al. (1967); Ball (1972) has also described individuals of *Blaberus craniifer* with secondary activity after dawn.

Harker (1956) showed that most of the feeding took place during the active period. Nevertheless, when food was offered in the light period only, the insects became active at this time as well as during the night. When feeding was discontinued, however, the daytime feeding peak did not persist and she concluded that the activity rhythm in *P. americana* was not an expression of a hunger cycle.

The locomotor activity rhythm in the house cricket, *Acheta domesticus* is similar to that in cockroaches. Lutz (1932) showed that activity commenced soon after dark and continued for about 4 to 6 hours. As with cockroaches, however, the pattern of activity varied between individuals and with age. Nowosielski and Patton (1963), for example, showed that last instar larvae rarely showed a pronounced rhythm, and that some adults were bimodal with a second peak prior to the onset of dark. Cymborowski (1973) demonstrated three types of individual in LD 12:12: some commenced activity at the light/dark transition, some commenced activity up to 3 hours after dark, and some began their period of intensified activity as much as 1 hour before light-off.

The activity rhythms of several mosquito species were recorded by automatic devices in which flight noise was amplified (Jones, 1964; Nayar and Sauerman, 1971). In LD 12:12 *Anopheles gambiae* was nocturnal but with an intense activity lasting 20 to 30 minutes following both light-off and light-on (Jones et al., 1966; Jones et al., 1967). *Aedes taeniorhynchus* was also a night-active insect with a similar bimodal pattern (Nayar and Sauerman, 1971). In this species the activity pattern originated in the adult instar and is not carried over from the developmental stages. The yellow-fever mosquito *A. aegypti*, however, was a diurnal insect with a main peak of activity about 1 to 2 hours before light-off and little or no activity in the dark portion of the cycle (Taylor and Jones, 1969). A bimodal pattern with a smaller peak following dawn was also apparent in this species.

The tsetse fly *Glossina morsitans* was found to be strictly diurnal. In a rocking actograph at LD 12:12 its activity occurred during the light in short bursts of about 1 minute duration separated by long intervals (Brady, 1970, 1972). Nevertheless, the mean hourly activity of groups of insects (teneral unfed males) revealed a clear V-shaped diurnal pattern with peaks in

the morning and evening, similar to that observed in the field; activity during the dark was almost negligible. The question of bimodality in activity rhythms, especially in mosquitoes and flies, will be re-examined in later sections, particularly with respect to its endogeneity.

In most insect studies, the so-called sleep-wake cycle is recorded by registering activity (= wakefulness) in such devices as running wheels, rocking actographs or by the disruption of an infra-red light beam; 'sleep' is then merely the *absence* of such activity. In honey bees (*Apis mellifera*) however, a behavioural state resembling *true sleep* (as in mammals and birds) has been described (Kaiser, 1988; Kaiser and Steiner-Kaiser, 1983). At night, prolonged rest is characterised by reduced muscle tone, decreased motility, lowered body temperature and a distinct posture in which the antennae droop. Nocturnal 'sleep' lasts for 5 to 8 h and is truly circadian, being regulated by endogenous (see below) rhythms emanating from the optic lobes. A sleep-like state has also been described in the fruit fly *Drosophila melanogaster* (Hendricks et al., 2000; Shaw et al., 2000).

B. THE ENDOGENOUS NATURE OF ACTIVITY RHYTHMS

1. 'Free-running' behaviour in the absence of temporal cues

Rhythms of activity in a light-dark cycle provide few clues as to the physiological nature of the controlling mechanism, which might have both endogenous and exogenous components. The endogenous nature of a rhythm, however, is usually revealed when the organism is transferred from a light-dark cycle (LD) into continuous dark (DD) or continuous light (LL), provided that temperature and other possible *Zeitgeber* are also held constant (Aschoff, 1960). Under these conditions an endogenous oscillation controlling a rhythmic activity will 'free-run' and reveal its natural periodicity (τ). In this state τ usually deviates slightly from 24 hours so that the onset or peak of activity appears either earlier or later by a few minutes every day. The fact that τ is close to but rarely equal to 24 hours is powerful evidence for an endogenous oscillator which is uncoupled from the environment and not being 'driven' by any uncontrolled *Zeitgeber* associated with the solar day.

Roberts (1960) studied the free-running rhythms of locomotor activity in the cockroaches *Leucophaea maderae*, *Byrsotria fumigata* and *Periplaneta americana*. When transferred to DD he found that the rhythms persisted for at least 3 months at a constant temperature of 25°C. In the three species studied τ for individual cockroaches varied between about 23 and 25 hours. The value of τ for an individual was not absolutely fixed, however, and in some instances was observed to change abruptly and spontaneously. A similar phenomenon has been observed in the cockroach *Nauphoeta cinerea* (Fig. 2.1) and the blow fly *Calliphora vicina* (Fig. 2.2.). Although τ varies between individual insects of the same species, the *range* of realisable τ values is almost certainly genotypic. In some individuals, a naturally occurring arrhythmicity in a particular behaviour may be found (Smith, 1987; Smietanko and Engelmann, 1989; Hong and Saunders, 1998).

Early authors (Gunn, 1940; Harker, 1956) reported a gradual loss of rhythmicity in cockroaches after a few days in constant light (LL). Roberts (1960), however, found no such loss for at least 20 days in *L. maderae* and for up to 7 weeks in *B. fumigata*. The difference between these results was attributed to the type of recorder used; in Harker's work, for example, tying the cockroach to a kymograph might have promoted a breakdown in activity not observed in a running wheel. Roberts (1960) also found that a transfer of the insects to LL caused τ to lengthen by between 20 and 60 minutes, and a second peak of running to occur

about 10 hours after the onset of primary activity. After transfer to DD this secondary peak disappeared.

The appearance of bimodality in LL and the spontaneous changes of τ in DD, both observed by Roberts (1960) in the cockroaches *L. maderae* and *B. fumigata*, are of particular interest because they indicate the probable multioscillator nature of the circadian system controlling activity rhythms (see Chapter 6). Both probably represent changes in the internal phase-relationship, or in the coupling, between constituent subsystems.

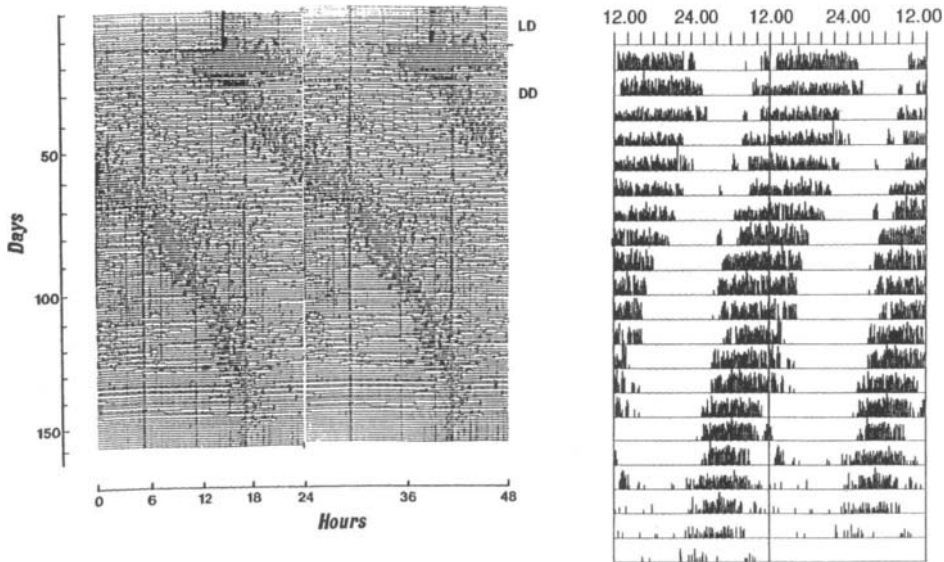


Fig. 2.1.(Left). Free-running locomotor activity rhythm in a male cockroach, *Nauphoeta cinerea*. The insect was kept in LD 15:9 for the first 13 days and then released into continuous darkness (DD). In LD locomotor activity was entrained and restricted to the early hours of the night; after transfer to DD the rhythm free-ran with an endogenous period (τ) less than 24 hours, showing several spontaneous shortenings of τ before death on the 158th day. In this, and all later and similar, actograms, insect activity is shown as dark 'bands' 'double-plotted' across the abscissa to aid visual inspection of the record. Each day's activity is stacked vertically. (Original).

Fig. 2.2. (Right). Free-running locomotor activity in an adult female blow fly, *Calliphora vicina*, in continuous darkness (DD) at 20°C with an endogenous period less than 24 hours ($\tau = 22.3$ h). (Original).

The free-running behaviour of a circadian rhythm in constant conditions has been observed in a large number of insect species and is now regarded as a universal property of the endogenous system. A few examples of such persistence will be mentioned here. Working with the house cricket *Acheta domesticus*, Lutz (1932) observed the persistence of the rhythm of locomotor activity in DD and concluded that it was endogenously controlled. Nowosielski and Patton (1963) later observed that the rhythm persisted for at least 2 weeks in both DD and LL.

Sokolove (1975) studied rhythms of locomotion and stridulation in males of the cricket *Teleogryllus commodus*. Both rhythms were circadian and free-ran in DD and in LL; τ was the same for both rhythms, in DD (23.4 hours) and in LL (25.1 hours). Although a slight overlap between the active phases of the two behaviours was noted in some cases, they were generally quite separate, locomotory activity occurring during the subjective day and stridulation at night. In this study there was no evidence to suggest that the two behaviours were controlled by

separate oscillators (although, for a later view, see Wiedenmann and Loher, 1984, and Wiedenmann et al., 1988). Loher (1979) extended this work to various circadian components of the behaviour and physiology of females of *T. commodus*. In LD cycles the majority of females ran in the dark component of the cycle (57 per cent) although many showed some locomotor activity in the 'day'. Eighty per cent laid their eggs in the light, however, and only 20 per cent in the dark. Taking both males and females of this species, there was a clear temporal correlation between the various reproductive behaviours: male and female locomotion, stridulation, spermatophore production and oviposition.

Page and Block (1980) extended studies on locomotor activity rhythms of cockroaches to stages other than the adult males. In *Leucophaea maderae*, first to fourth instar nymphs were shown to possess activity patterns similar to those of adults but with significant changes in τ during development, and regular fluctuations associated with the moulting cycle. In constant darkness, τ for female adults was slightly but consistently longer than that for males. Page (1990) showed that about 40 per cent of nymphs of *L. maderae* were rhythmic in DD, with a mean period (τ) of about 23.7 h.

In another Orthopteran, the large flightless New Zealand weta, *Hemideina thoracica*, free-running locomotor rhythms have been recorded for months in DD and in LL showing, among other properties, striking spontaneous changes in τ (Lewis, 1976; Christensen and Lewis, 1982) (Fig. 2.3). Extensive studies of the circadian system in *H. thoracica* have led to the development of a useful, predictive and realistic feed-back model for circadian rhythmicity (see Christensen and Lewis, 1983; Christensen, Lewis and Gander, 1984; Chapter 7).

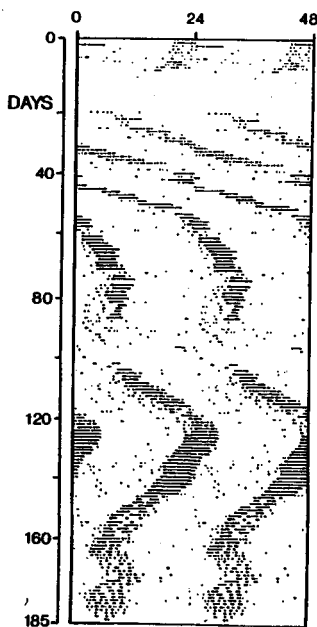


Fig. 2.3. Locomotor activity rhythm in the weta, *Hemideina thoracica*, free-running in DD, showing several spontaneous changes in τ . (From Christensen and Lewis, 1982).

Further examples of free-running and therefore endogenous circadian rhythms of behaviour have been recorded in numerous species from many insect orders: this leads us to suppose that such innate rhythmicity is almost universal. In nearly every case the rhythm persists with a circadian periodicity in DD, but the effects of constant light (LL) of different intensities may be various. One exception to this rule seems to be the blow fly *Protophormia terraenovae* which, although showing a free-running rhythm of locomotor activity in LL above about 1 lux, shows total *inactivity* in LL below that intensity and, surprisingly, also in DD (Aschoff and von Saint Paul, 1982). In some species, and at some intensities, overt rhythmicity 'damps-out' or apparently stops in LL; these effects will be considered below (Section B.3(b)).

2. Insects living in habitats with little or no environmental periodicity

Since insects and other animals living in habitats with a well-defined periodicity of light and darkness, or of temperature, show clear and usually endogenous rhythms of activity, it is of interest to investigate locomotor activity rhythms of insects living in areas devoid or deficient in such environmental fluctuations. For example, insects living in caves or inside logs may be in continuous darkness or in habitats with little diurnal fluctuation in light intensity and temperature; conversely, those inhabiting higher latitudes may be living in conditions approaching 'constant light' during the arctic summer.

Animals in caves may be divided into three broad groups: troglobites or obligate cavernicoles, troglaphiles or facultative cavernicoles; and troglonexes or animals that return periodically to the surface and hence live in the cave only part of the time. Insect examples from each of these groups have been examined for their circadian rhythmicity, and these activity patterns seem to reflect their way of life. The cave crickets *Hadenoeus subterraneus* (Reichle et al., 1965) and *Ceuthophilus conicaudus* (Campbell, 1976), for example, are troglonexes: they spend their days within the cave, but emerge at night to feed. These species possess well-defined circadian rhythms of locomotion and associated behaviours. *H. subterraneus* maintained in the laboratory at 13°C and in constant darkness showed a clear circadian activity rhythm for 23 days. *C. conicaudus* had a similar persistent rhythm in DD (21°C), with activity strongly reduced in the light. In neither species, however, is there any information on the free-running period τ .

Lamprecht and Weber (1977) examined the activity rhythms of three species of beetle, the troglaphiles *Laemostenus terricola* and *L. oblongus*, and *L. navarricus* which is an obligate cavernicole or troglobite. The two troglaphiles possessed reasonably well-defined activity rhythms in DD and in LL. In *L. navarricus*, however, rhythms were very 'imprecise' and were 'extinct' in DD. *L. navarricus* was considered to be adapted for life in the total darkness of the deeper parts of the cave, whereas the troglaphiles were adapted to the 'twilight zones' near the cave entrance.

Insects living within the trunks of trees or within fallen logs experience a similar paucity of diurnal fluctuation. Park (1937) demonstrated that the log-inhabiting beetle *Passalus cornutus* was arrhythmic in its natural habitat and in constant laboratory conditions, activity being 'uninfluenced' by DD, LL, or LD. The cerambycid *Rhagium inquisitor* whose larvae live in the rotting trunks of pine trees, on the other hand, showed a weak daily rhythm of feeding and digestion apparently entrained by the temperature cycle (thermoperiod) but not by light (Riba, 1976).

The converse situation of environments with continuous illumination may be experienced by insects living in high latitudes, although the 'constant light' of the subarctic summer contains an appreciable diurnal fluctuation in light intensity and temperature. In

Swedish Lapland (66°N), Müller (1973) showed that insects living in running water (larvae of mayflies, stoneflies and *Simulium* spp.) may be asynchronous or arrhythmic in their behaviour patterns, whereas air-living organisms such as emerging and flying insects were rhythmic and entrained to the weak solar-day *Zeitgeber*. Several species of carabid beetle, including *Carabus violaceus*, on the other hand, showed activity at all times of the arctic day, at best with a weak rhythmicity little enhanced by an experimental LD cycle (Hempel and Hempel, 1959). Although these observations say little about the circadian system as a whole, it would appear that insects only retain a functional circadian clock when their natural history demands it. Trogloneic crickets, for example, possess a well-developed clock to enable them to 'anticipate' dusk and time their nocturnal exits from the cave. Deep in the aphotic zones of caves or in the continuous light of midsummer subarctic waters, on the other hand, there may be little or no selective advantage in possessing a clock, and insects are essentially arrhythmic.

3. The effects of constant light (LL)

(a) 'Aschoff's rule' and the 'circadian rule'

The range of τ values observed for a group of *Leucophaea maderae*, and the increase in τ on transfer from DD to LL are well illustrated in Fig. 2.4 (Lohmann, 1967). This change in circadian period is in accordance with 'Aschoff's rule' (Pittendrigh, 1960), which states that τ lengthens with an increase in light intensity, or on transfer from DD to LL, for dark-active animals (i.e. $\tau_{DD} < \tau_{LL}$, nocturnal), but *shortens* for light-active animals (i.e. $\tau_{DD} > \tau_{LL}$, diurnal). An example of the effects of LL on locomotor activity in *Nauphoeta cinerea* is shown in Fig. 2.5. For insects, however, the *general* applicability of this 'rule' is in doubt (Table 2. 1). In some nocturnal species such as cockroaches (Roberts, 1960), the house cricket *Acheta domestica* (Nowosielski and Patton, 1963), the pond skater *Velia currens* (Rensing, 1961), and the flour beetle *Tenebrio molitor* (Lohmann, 1964) the results for a transfer from DD to LL, and vice versa, are generally in agreement with 'Aschoff's rule'. In cockroaches, however, Roberts (1960) found no obvious correlation between τ and the intensity of illumination (in LL) and, in *V. currens*, Rensing (1961) failed to observe a systematic change of τ when the intensity was raised from 0.1 to 700 lux. Furthermore, in the mainly light-active dung beetle, *Geotrupes sylvaticus*, Geisler (1961) suggested that τ lengthened in LL.

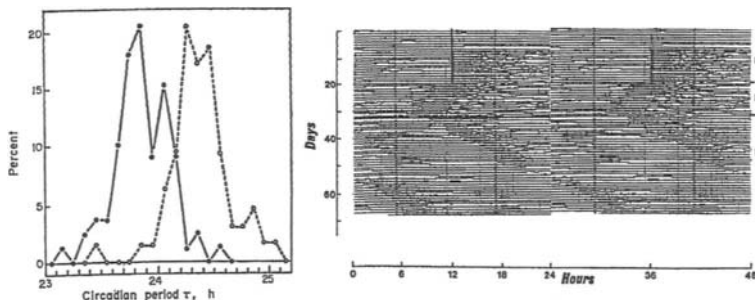


Fig. 2.4.(Left). Relative frequency distributions of free-running periods (τ) of *L. maderae* in DD (closed circles; $N = 77$) and in LL (open circles; $N = 64$). (Redrawn from Lohmann, 1967).

Fig. 2.5. (Right). Locomotor activity rhythm of a male cockroach, *Nauphoeta cinerea*, showing an increase of τ on transfer from DD to LL ($0.016 \mu W cm^{-2}$), and an appearance of 'bimodality' in LL. (Original, from Thomson, 1976).

Youthed and Moran (1969a) described a circadian rhythm of nocturnal pit-building activity by larvae of the ant-lion *Myrmeleon obscurus* with a peak in activity soon after dusk. This rhythm persisted in both LL and DD for at least 1 month. In continuous light τ varied from 23 hours 44 minutes to 23 hours 59 minutes; in DD it was between 24 hours 2 minutes and 24 hours 17 minutes. This lengthening of τ in DD for a nocturnal species clearly violates ‘Aschoff’s rule’. Taylor and Jones (1969) also demonstrated that τ increased in LL in the day-active mosquito *Aedes aegypti*. Weber (1967) studied the effects of constant light on three species of *Carabus*, *C. problematicus*, *C. cancellatus* and *C. nitens*. In the first of these he showed a non-monotonic relationship between τ and light intensity, with a maximum between 1.5 and 4.0 lux (Table 2.1).

TABLE 2.1. Circadian period (τ) of overt locomotor activity rhythms in insects showing changes in τ on transfer from DD to LL, or with an increase in light intensity. Asterisks mark those examples that appear to violate ‘Aschoff’s rule’. N = nocturnal; D = diurnal.

Species	N/ D	τ_{DD} hours	τ_{LL} hours	Reference
<i>Leucophaea maderae</i>	N	23.4-24.0	24.0-24.75	Roberts (1960)
<i>Byrsotria fumigata</i>	N	23.9-24.4	24.4-25.5	Roberts (1960)
<i>Periplaneta americana</i>	N	23.8	24.5	Roberts (1960)
<i>Nauphoeta cinerea</i>	N	23.73	24.56 at 0.016 Wm ⁻² 24.65 at 0.018 Wm ⁻²	Thomson (1976)
<i>Hemideina thoracica</i>	N	24.7	Up to 31.8 in 1 lux	Christensen (1978)
<i>Teleogryllus commodus</i> male	?	23.4-23.5	25.1-25.6	Loher (1972)
<i>Acheta domestica</i>	N	< 24	> 24	Sokolove (1975)
<i>Tenebrio molitor</i>	N		24.3 at 0.01 lux 25.08 at 2 lux 26.07 at 100 lux	Nowolsielski and Patton (1963)
<i>Geotrupes sylvaticus</i> *	D	24	> 24	Lohmann (1964)
<i>Carabus problematicus</i>	N	23.07	26.0 at 1.5 lux 25.6 at 4 lux 24.6 at 12 lux 24.3 at 55 lux 23.9 at 150 lux 23.6 at 250 lux	Giesler (1961)
<i>Myrmeleon obscurus</i> *	N	24 – 24.2	23.75 – 24.0	Weber (1967)
<i>Aedes aegypti</i>	D	22.5	26.0	Youthed and Moran (1969a)
<i>Culex pipiens fatigans</i>		~ 24	~ 26	Taylor and Jones (1969)
<i>Calliphora vicina</i> *	D	22.95	24.61 at 0.035 Wm ⁻²	Jones (1976)
<i>Drosophila melanogaster</i> *	D			Hong and Saunders (1994)
per ^S		~19.0	Shortens up to 0.1 lux	Konopka et al. (1989)
per ^L		~29.0	Lengthens up to 0.1 lux	

One difficulty with the application of Aschoff’s rule to insects concerns the decision whether a particular species is nocturnal or diurnal. For example, many mosquitoes and species

of *Drosophila* are crepuscular in habit. Sokolove (1975) also pointed out that males of the cricket *Teleogryllus commodus* are day-active with respect to their locomotor activity but stridulate at night. Females, on the other hand, are mainly night-active in their locomotion: it is at this time that they actively seek singing males. Males of this species therefore 'obey' Aschoff's rule for stridulatory activity, but not for locomotion.

In the New Zealand weta, *Hemideina thoracica*, Christensen (1978) and Christensen and Lewis (1982) showed that τ increased after transfer from DD to LL (0.1 lux) at all temperatures tested, but this increase was greatest at 16°C (3.5 to 5.3 hours) and least at 27°C (0.1 to 1.2 hours).

Aschoff (1960) later extended the so-called 'Aschoff's rule' to cover two more parameters: (1) the ratio of activity-time (α) to rest-time (ρ), and (2) the total amount of activity per circadian cycle. For a number of vertebrate species kept in constant conditions, Aschoff showed that both of these parameters increased with increasing light intensity in day-active animals, but decreased with light intensity in night-active animals. The generalisation based on these observations - called the 'circadian rule' - has been used in a model for circadian oscillations which suggests that the rhythm of locomotor activity is based on a continuous function which crosses a threshold twice during each daily cycle, and that activity only occurs when the function is above the threshold (Wever, 1965). Nevertheless, although this 'circadian rule', like 'Aschoff's rule', holds good for a number of birds and mammals (Hoffmann, 1965), it is violated by several examples from the insects. In particular, Lohmann (1964) showed - for the nocturnal beetle *Tenebrio molitor* - a positive correlation between the $\alpha:\rho$ ratio and light intensity - despite the fact that the increase of τ on transfer from DD to LL agreed with 'Aschoff's rule'. Other violations of the circadian rule were observed for the cockroaches *Byrsotria fumigata* and *Leucophaea maderae* (Roberts, cited in Hoffmann, 1965).

Locomotor and flight activity rhythms frequently become arrhythmic in LL at higher light intensities. Examples include flight activity of male *Lucilia cuprina*, which becomes arrhythmic above about 5 lux (Smith, 1983), and locomotor activity in females of *Calliphora vicina* (Hong and Saunders, 1994). In *C. vicina*, τ increased from about 22.5 hours in DD to as much as 25.6 hours in LL (up to 0.03 Wm⁻²). Under still brighter light (>0.03 Wm⁻²) locomotor activity became random (Fig. 2.6). In some flies at an 'intermediate' intensity (~0.024 Wm⁻²), activity was initially arrhythmic but then became rhythmic with a longer period as if their photoreceptors had become 'light adapted'.

Working with wild type and *period* mutants of *Drosophila melanogaster* (see Chapter 4), Konopka et al. (1989) showed that the free-running period of wild type (Canton-S) flies increased by as much as 10 to 11 hours as light intensity increased from 0.001 to 0.1 lux, but behaviour became arrhythmic above about 1 lux. The periods of both the short period mutant (*per^S*; τ about 19 hours) and a long period mutant (*per^{L1}*; τ about 29 hours) also increased in LL but arrhythmicity occurred at a lower intensity than in wild type, suggesting that mutation at the *period* locus also altered photosensitivity. In the case of *per^{L1}*, τ increased by as much as 16 to 18 hours before arrhythmicity ensued.

(b) Does constant light (LL) 'stop' the clock?

Many 'physiological' or 'developmental' rhythms such as pupal eclosion (see Chapter 3) free-run in DD but seem to damp out quite rapidly in LL of quite low intensity. In addition they frequently appear to be reset close to a unique phase (called Circadian time, Ct 12) at, or soon after, a subsequent return to darkness. As we have seen above, however, many locomotor or 'behavioural' rhythms - with some notable exceptions, principally among flies (Jones, 1973;

Smith, 1983; Konopka et al., 1989; Hong and Saunders, 1994) – seem to free-run in LL of quite high intensity as readily as they do in DD. This difference between ‘developmental’ and ‘behavioural’ rhythms was one of the features used by Truman (1971d) to distinguish his Type I from Type II clocks (Chapter 6, B.5). But since some ‘behavioural’ rhythms are now known to ‘stop’ in LL of quite low intensity, this phenomenon might merely be one of photoreceptor sensitivity. The question of whether constant light ever really ‘stops’ circadian oscillators was addressed by Peterson and Jones (1979) with reference to the flight activity rhythm in the mosquito *Culex pipiens* and will be discussed further in Chapter 3.

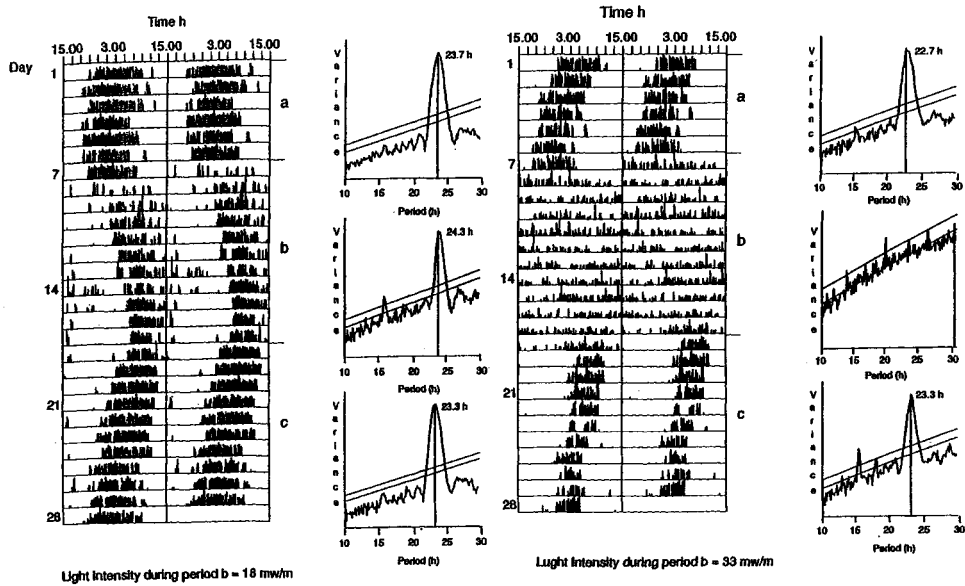


Fig. 2.6. The effect of constant light (LL) on the locomotor activity rhythm of female blow flies (*Calliphora vicina*) at 20°C. The flies were initially in DD, then in LL of different intensity, and finally back to DD. Left – LL of low intensity (0.018 Wm^{-2}) causing an increase in τ ; Right – LL of a higher intensity (0.033 Wm^{-2}) leading to behavioural arrhythmicity. (From Hong and Saunders, 1994).

Resetting of the circadian oscillator to a unique phase (equivalent to Ct 12, or the beginning of the subjective night) when transferred from LL to DD will also be dealt with more fully in Chapter 3 when describing properties of rhythms such as pupal eclosion. Among locomotor rhythms such behaviour has been noted with flight activity in *Lucilia cuprina* (Smith, 1983), but was not apparent with locomotor activity in *Calliphora vicina* (Hong and Saunders, 1994).

4. Homeostasis of the natural circadian period (τ)

(a) Temperature compensation

Roberts (1960) showed that the period of the locomotor rhythm in *Leucophaea maderae* was temperature-compensated. In one individual, for example, τ shortened from 25

hours 6 minutes at 20°C to 24 hours 24 minutes at 25°C and to 24 hours 17 minutes at 30°C. Although the calculation of a temperature coefficient was complicated by the known lability of τ in individual cockroaches, the Q_{10} was estimated to be about 1.04 for the rise in temperature from 20° to 30°C. In the weta, *Hemideina thoracica*, estimates of Q_{10} for the locomotor rhythm varied from 0.82 to 1.15 (Gander, 1976). Working with *L. maderae*, Caldarola and Pittendrigh (1974) showed that τ was a non-monotonic function of temperature. It was greatest at 17°C, least at just over 20°, and greater again at 30°; the slope of the curve was negative at 20° but positive at 30° (Fig. 2.7B). In an earlier paper, Pittendrigh and Caldarola (1973) transferred *L. maderae* (maintained in DD) from 20 to 30°, and from 30 to 20°. In the former, τ lengthened from 23.05 to 23.69 hours, a Q_{10} of 0.97, but in the latter it shortened from 23.72 to 22.94, showing an overshoot of the previous value at 20°. The range of τ -values at 30° was also smaller than at 20°C. Wiedenmann (1978) found τ for *L. maderae* to be 22.8 hours at 20°, and 23.4 hours at 28°, a Q_{10} of 0.97.

In a more recent example concerning locomotor rhythmicity of the blow fly *Calliphora vicina* (Fig. 2.7A) Saunders and Hong (2000) showed that steps-up (in DD) from 20 to 25°C gave a mean Q_{10} of 0.98, whereas down-shifts from 20 to 15°C, or from 25 to 15°, gave Q_{10} s of 1.04 and 1.03. Values for Q_{10} close to 1.0 reveal the virtual 'independence' of the free-running oscillation to temperature, a fundamental property of circadian rhythms which gives them their functional significance as a time-measuring system (Pittendrigh, 1960) (see Table 2.2).

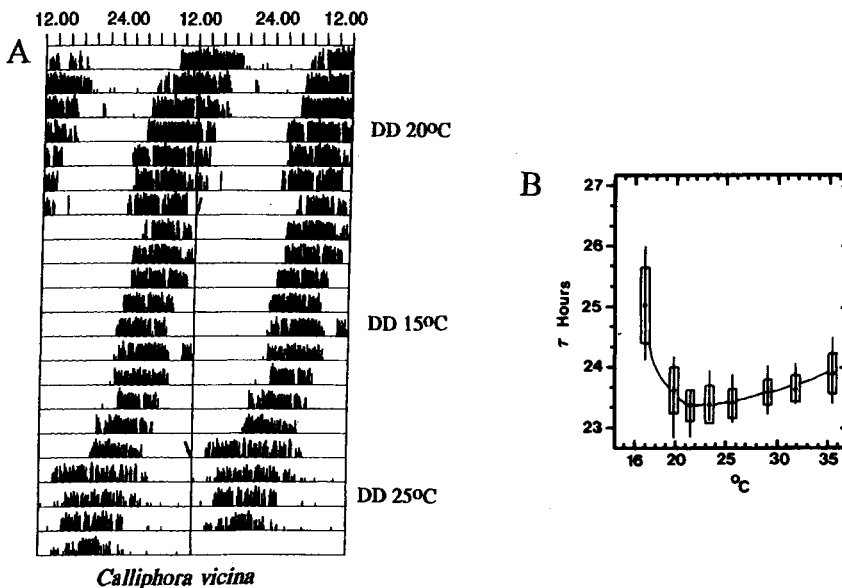


Fig. 2.7. Temperature compensation of the free-running rhythm in DD. A – Locomotor activity rhythm of a female blow fly (*Calliphora vicina*) days 1 to 7 at 20°C, then days 8 to 18 at 15°C, and finally to 25°C, all in DD. (From Saunders and Hong, 2000). B - *Leucophaea maderae*. Locomotor activity rhythm in DD, showing the non-monotonic relationship between temperature and the free-running period, τ . The standard deviations of τ are represented by the rectangles around τ , and the range of τ values by the vertical line through the mean. N = 7 to 10 for each temperature. (From Caldarola and Pittendrigh, 1974).

In a study of wild-type and *period* mutants of *Drosophila melanogaster* (see Chapter 4), Konopka et al. (1989) showed that mutation at the *period* locus had profound effects on temperature compensation as well as circadian period. For example, although τ_{DD} for Canton-S wild type was about 24.15 hours at 17° and 23.82 hours at 25°, τ_{DD} for short-period flies (*per^S*) shortened from 19.54 to 18.7 hours, but that for long-period flies (*per^L*) lengthened from 27.81 to 30.54 hours over the same temperature range. Short and long period flies thus exhibited reciprocal behaviour with respect to the dependence of τ on temperature (Fig. 2.8).

TABLE 2.2. Temperature compensation. Approximate temperature coefficients (Q_{10} values) for the free-running circadian period in darkness (τ_{DD}) in various insects. A Q_{10} value of less than one indicates ‘over-compensation’ in which τ_{DD} increases after transfer to a higher temperature

Species	Temperature range	Q_{10} values	Reference
<i>Leucophaea maderae</i>	20 → 30 °C	1.04	Roberts (1960)
	20 → 30 °C	0.97	Pittendrigh and Caldarola (1973)
	20 → 28 °C	0.97	Wiedenmann (1978)
<i>Hemideina thoracica</i>	various	0.82-1.15	Gander (1976)
<i>Drosophila melanogaster</i>			
<i>per⁺</i>	17 → 25 °C	1.01	Konopka et al. (1989)
<i>per^S</i>	17 → 25 °C	1.07	
<i>per^L</i>	17 → 25 °C	0.86	
<i>Calliphora vicina</i>	20 → 25 °C	0.98	Saunders and Hong (2000)
	25 → 15 °C	1.03	

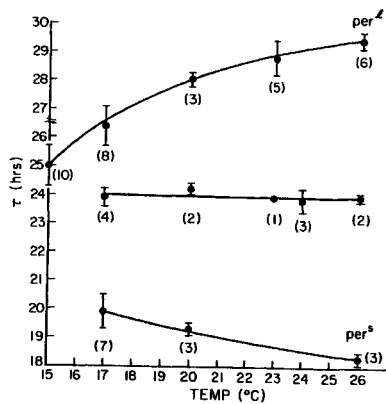


Fig. 2.8. *Drosophila melanogaster*. The periods of locomotor activity rhythms of adult wild-type and *period* mutant flies, measured under constant infrared light. The number of flies used for each point is given in parentheses. *Per^L* long period mutant; *per^S* short period mutant (From Konopka, Pittendrigh and Orr, 1989).

(b) *The effects of D₂O and lithium*

In a range of organisms from the unicellular alga *Euglena gracilis* (Bruce and Pittendrigh, 1960) to the rodent *Peromyscus leucopus* (Suter and Rawson, 1968) heavy water is known to exert dose-dependent lengthening effects on the circadian period in DD. In the cockroach *L. maderae*, Caldarola and Pittendrigh (1974) showed that D₂O administered in the drinking water at a concentration of 25 per cent lengthened τ at both 20 and 30°C, but the increases were comparatively slight (3.64 per cent at 20 °C, 1.30 per cent at 30 °C), like those noted for other organisms (Fig. 2.9).

Lithium salts also lengthen the circadian period in a number of organisms including the flowering plant *Kalanchoë blossfeldiana* (Engelmann, 1973), the hamster *Mesocricetus auratus* and the cockroach *Leucophaea maderae* (Hofmann et al., 1978). In the latter, lithium chloride was added to the drinking water of insects free-running in continuous red light. In twenty-two out of thirty-one cases there was an immediate increase in τ in the order of about 0.1 to 0.2 hours; in others there were either delayed lengthening effects, or no effect. In most insects there was a return to a shorter value of τ when the lithium was replaced by water.

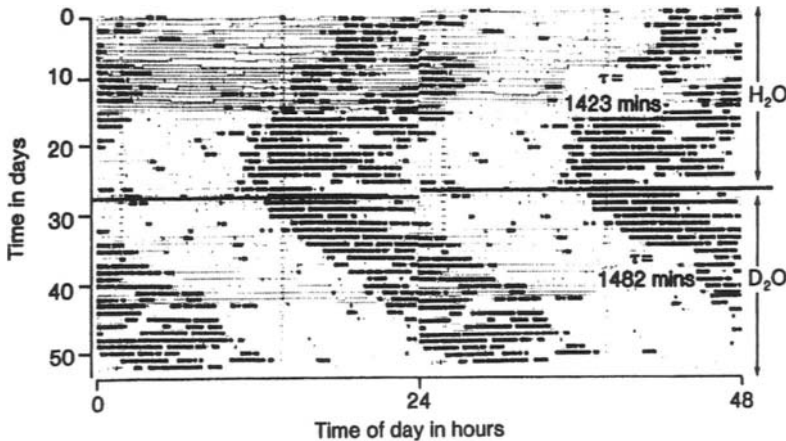


Fig. 2.9. The effect of deuterium oxide (25 per cent) in the drinking water on the free-running period of the locomotor rhythm in the cockroach, *Leucophaea maderae*, showing an immediate increase in τ (From Caldarola and Pittendrigh, 1974).

The importance of these observations on the period-lengthening effects of temperature, D₂O and lithium, is two-fold. At this juncture it is sufficient to note that the effects are slight: far less, for example, than the effects of temperature or D₂O on 'non-clock' or temperature-dependent processes (Pittendrigh et al., 1973). The conclusion must be that this phenomenon reflects a general homeostasis of the period of the circadian pacemaker(s), to any changes in the cellular milieu that might render time measurement impossible. For example, if τ were subject to a doubling or trebling with a 10° rise in temperature - which is 'normal' for most physiological processes - the system would be useless for 'telling the time'. The free-running period would also soon fall outside the range of entrainment by the solar-day *Zeitgeber* (Section E.2) and another important aspect - control of phase-angle - would cease to function.

The second importance of these observations is a physiological one concerning the possible nature of the circadian pacemaker (see Chapter 4).

Administration of 1mM lithium chloride to the drinking water of adult house flies (*Musca domestica*), maintained throughout the experiment in constant light (0.5 lux), frequently caused period lengthening, but also the appearance of 'compound' rhythms or arrhythmia (Smietanko and Engelmann, 1989). Schmid and Engelmann (1987) also showed period lengthening in *M. domestica* with both lithium and rubidium. With Rb^+ the degree of lengthening was dependent on initial period: flies with a shorter period showed lengthening of τ , whereas those with a longer period showed shortening. The increase in the proportion of flies showing arrhythmia or 'compound' rhythms after Li^+ treatment was interpreted as an effect upon the mutual coupling between constituent oscillators within a complex pacemaker. This concept will be examined further in Chapter 6.

5. Bimodal activity patterns

Most of the insects taken in suction traps by Lewis and Taylor (1965) showed unimodal activity patterns. Others, however, particularly small species with crepuscular habits, were bimodal. Especially important in this respect are species of *Drosophila* and mosquitoes: both have attracted considerable attention in laboratory studies and will be examined here.

Many species of *Drosophila* show bimodal activity rhythms in field conditions with peaks close to dawn and dusk, and a suppression of activity around noon (Taylor and Kalmus, 1954; Dyson-Hudson, 1956; Lewis and Taylor, 1965; Hardeland and Stange, 1973). This midday 'resting period' is seen in species from non-arid as well as arid localities. In artificial light-dark cycles in the laboratory, *Drosophila* species may be bimodal (Hardeland and Stange, 1971; Engelmann and Mack, 1978) or unimodal (Roberts, 1956). Roberts showed that transfer of *D. robusta* from LD 12:12 to dim constant light resulted in the persistence of the single (dusk) peak of activity with a near 24 hours periodicity. In *D. melanogaster* adult activity also free-ran in constant conditions (infra-red light) with a mean circadian period of 24.5 ± 0.4 hours (Konopka and Benzer, 1971). In the normally bimodal *D. pseudoobscura*, Engelmann and Mack (1978) found that only the 'dusk' peak of activity persisted in DD ($\tau = 21.3$ to 23.9 hours), the 'dawn' peak apparently being an exogenous effect of 'lights-on' (Fig. 2.10).

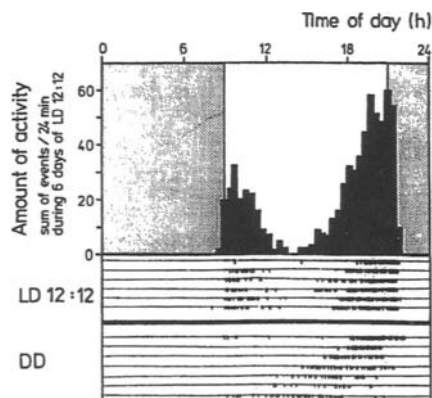


Fig. 2.10. Locomotor activity rhythm of adult *Drosophila pseudoobscura* showing bimodality in LD 12:12, but only the 'dusk' peak persisting ($\tau < 24$ hours) in subsequent DD free-run. (From Engelmann and Mack, 1978).

Hamblen-Coyle et al. (1992) examined activity profiles of wild type and *period* mutants of *D. melanogaster* (see Chapter 4) in light-dark cycles (LD 12:12). Wild type flies showed a typical bimodal pattern with 'dawn' (morning) and 'dusk' (evening) peaks of activity, and phases of relative inactivity in the middle of the day and night. Flies 'anticipated' dawn and dusk with an increased level of activity; this indicated the endogeneity of both components. Short-period mutants (*per^S*, $\tau_{DD} \sim 19$ hours) showed an earlier occurrence of the evening activity peak and long-period flies (*per^{L2}*, $\tau_{DD} \sim 29$ hours) showed a delayed second peak. The positions of the morning peak, however, were similar in both mutants and in wild type (Fig. 2.11). Unlike the case of *D. pseudoobscura* (Engelmann and Mack, 1978), both peaks free-ran in subsequent DD, thereby indicating their endogeneity. The phase angles (ψ) between the peaks of activity and the light-dark cycle were interpreted by reference to phase response curves (see section E2). Helfrich-Förster (2000) showed that the majority of wild-type flies (Canton-S, Berlin and Oregon-R) showed bimodality under LD conditions with clear morning (M) and evening (E) peaks but a sexual dimorphism in phase, the morning peak in males occurring significantly earlier than in females. In continuous darkness, M merged with E in about half the flies to give a unimodal activity band, but in others the two components desynchronised indicating two separate oscillators.

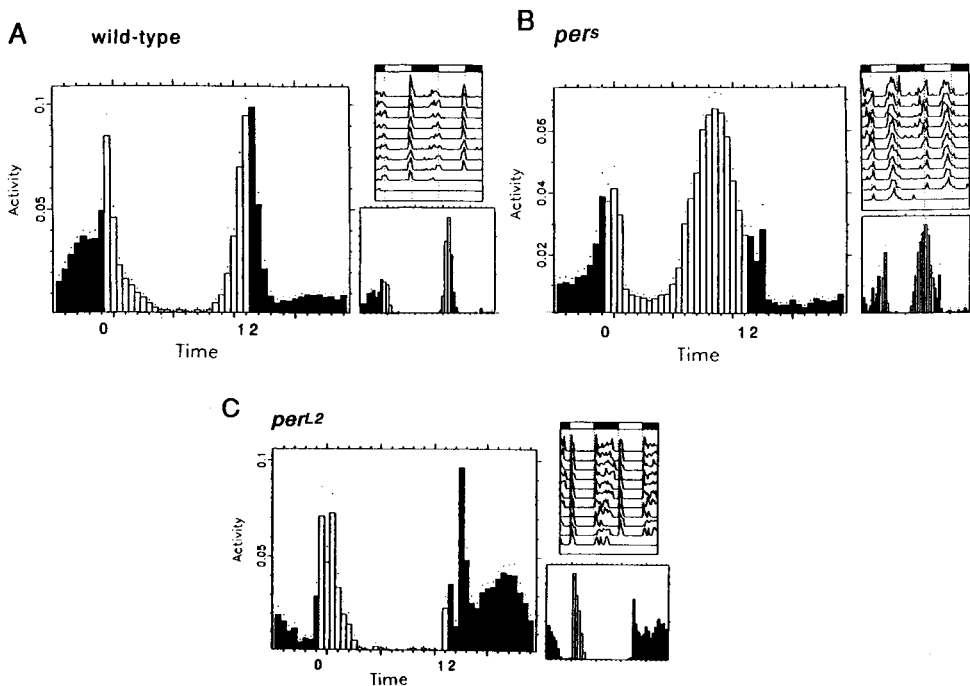


Fig. 2.11. Bimodality in the locomotor activity rhythms of wild type and *period* mutants of *Drosophila melanogaster* entrained to a light cycle (LD 12:12). A – wild type; B – short period mutant, *per^S*; C – long period mutant, *per^{L2}*, showing phase relationships of the 'morning' and 'evening' peaks of activity to dawn and dusk. (From Hamblen-Coyle et al., 1992).

Wheeler et al. (1993) showed that 'arrhythmic' (*per*⁰) mutants of *D. melanogaster* were rhythmic in LD cycles, but this periodicity was considered to be 'forced' (i.e. exogenous) rather than a consequence of any remaining oscillation(s). In a more recent paper, however, Helfrich-Förster (2001) showed that *per*⁰ flies retained the morning (M) oscillator but lost the evening (E) peak. In most cases the M component was not 'strong' enough to drive the rhythm in DD, but in LD the activity of *per*⁰ flies was entrained. She suggested that the phase of E was affected by mutation at the *per* locus and was entrained by light through the agency of *cryptochrome* (see Chapter 4). The morning peak, however, was *independent* of *per* and mainly entrained by light through the compound eyes (Emery et al., 2000). These important observations have relevance when considering the multioscillator nature of the circadian system regulating locomotor rhythmicity (see Chapter 6), and the nature of the photoperiodic oscillator in *D. melanogaster* and possibly other species (see Chapter 13). The question of periodicity in apparently arrhythmic *per*⁰ flies will be considered further in Chapters 6 and 16.

Other types of insect may show bimodality in their locomotor activity rhythms under certain conditions. Amongst the cockroaches, for example, some individuals may show bursts of activity following dawn and dusk (Lipton and Sutherland, 1970; Ball, 1972), although only the latter usually persists in DD. In constant light several authors have reported two activity periods (Roberts, 1960; Wiedenmann, 1977, 1980): the possible relationship of this phenomenon to 'splitting' will be considered later (Chapter 6).

Circadian flight-activity rhythms in mosquitoes have been intensively studied by automatically recording flight sounds (Jones, 1964). In many species these rhythms were clearly bimodal (see for example, Fig. 2.12). *Anopheles gambiae* is a nocturnal species with a pronounced peak of activity shortly after dusk and a second peak in the middle or later part of the night (Jones et al., 1967; Jones et al., 1972b). When transferred to DD the rhythm free-ran with a period (τ) close to 23 hours; constant light inhibited flight activity. In lighting regimes with an abrupt onset of light there was an additional dawn peak of activity which disappeared when the abrupt transition was replaced by a more gradual one simulating a natural dawn (Jones et al., 1972b). This peak was therefore thought to be an exogenous or 'startle' reaction associated with the abrupt onset of the light. Working with virgin females of *A. gambiae* in LD 12:12, Jones and Gubbins (1977) showed that the 'lights-off' or dusk peak of activity showed a sharp reduction after insemination, whereas the second nocturnal peak was enhanced. This behavioural change appeared to be associated with the fact that the first peak corresponded to the times of egress of mosquitoes from their daytime resting place, and of mating, whereas the latter peak occurred when the mosquitoes were actively host-seeking and biting. After a bloodmeal, on the other hand, there was an initial depression of activity for 2 to 3 days, then an increase in the relative importance of the first peak until oviposition had taken place (Jones and Gubbins, 1978).

In *A. gambiae* the lights-off activity peak was endogenous and free-ran in DD with τ 22.5 to 23 hours, but the length of the first period in DD was longer than that in free-run. This distortion, moreover, was a function of the duration (and intensity) of the final period of light: it was about 1 to 1.5 hours longer after a final light period of 12 hours, and was extended by a further hour after a 48-hour light (Jones, 1973). A possible explanation for this phenomenon may be found by supposing that the circadian system of *A. gambiae* consists, not of a single oscillation, but of a more complex hierarchical arrangement of 'pacemakers' and driven rhythms which respond differently to the extended periods of light. This interpretation will be examined further in Chapter 6.

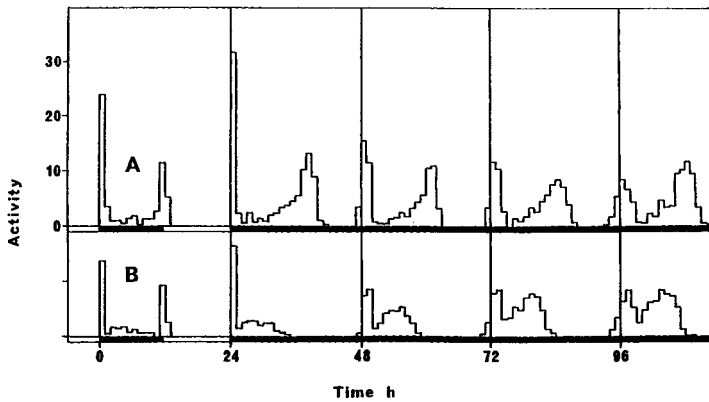


Fig. 2.12. *Culex pipiens quinquefasciatus*. Mean flight activity in LD 12:12 (one cycle) followed by DD. A – virgin females (N = 33) showing persistent bimodality in DD; B – inseminated females (N = 35) showing modification of the position of the ‘dusk’ peak. (From Jones).

Flight activity in the diurnal species *Aedes aegypti* was also bimodal with a small peak at light-on in addition to the main peak of activity about 1 to 2 hours before light-off. The rhythm persisted in DD with a low level of activity and a period (τ) of about 22.5 hours (Taylor and Jones, 1969). When adults were transferred from LD 4:20 to DD both the light-on and the light-off peaks persisted at the expected times indicating that both peaks were endogenously controlled. Since they appeared to be independently phase-set by the ‘on’ and ‘off’ signals they may represent two independent (‘dawn’ and ‘dusk’) oscillators (Taylor and Jones, 1969). The bimodal pattern of flight activity in *Aedes taeniorhynchus* also persisted in DD with a period of about 23.5 hours (Nayar and Sauerman, 1971).

In *Culex pipiens fatigans* the bimodal rhythm of flight activity persisted in DD with τ about 24 hours, but in LL (50 lux) the rhythm became unimodal with τ about 26 hours (Jones, 1976). Although the rhythm persisted in LL of this intensity, its phase was reset at a subsequent transfer to DD. As with *A. gambiae* (Jones, 1973) there was a distortion in the first period in DD, but in *C. pipiens* the period of the first cycle was found to be a cyclical function of the duration of the last light period. It was greatly lengthened, for example, when the final light period was 24 or 48 hours, but less after a light period of 12, 36 or 60 hours (Jones, 1976). The explanation for this ‘resonance’ effect is probably that the light to dark transition occurred at different circadian phases of the undamped oscillation in LL, and this led to phase shifts of different magnitude. This result is also relevant to a discussion of the probable multioscillator nature of the circadian system (Chapter 6).

Taylor (1969) examined the flight activity patterns of seven species of mosquito from the genera *Anopheles*, *Aedes* and *Culex*. Whilst most were bimodal in LD 12:12, more complex patterns were seen when the photoperiod departed from 12 hours. In *Anopheles farauti*, for example, he recognised three activity peaks, one after lights-off, one after lights-on, and one 11 to 13 hours after lights-off. When the photoperiod was between 11 and 13 hours, the second and third of these peaks seemed to coincide and reinforce each other to provide the characteristic bimodality; in the other photoperiods, however, all three peaks were evident. Taylor found a close, or reasonably close, correlation between the range of photoperiods giving rise to the coincidence between these two peaks, the latitudes at which these photoperiods

occur, and the known geographical distribution of the species. In *A. farauti*, for example, 11-hour to 13-hour photoperiods and its distribution are found to occur between 0 and 20°N.

In the tsetse fly *Glossina morsitans* the mean hourly activity of a group of teneral males showed a clear V-shaped pattern in LD 12:12 with peaks in the morning and the evening (Brady, 1972). In conditions of constant darkness the free-running pattern, compiled by synchronising the individual records to the time of appearance of their first major activity peak, showed that the rhythm persisted for at least 4 days. The endogenous nature of the rhythm in LL was less clear.

Brady and Crump (1978) showed that the V-shaped pattern of activity was similar in field and laboratory. The principal *Zeitgeber* appeared to be dawn: a 6-hour advance in the lights-on signal leaving 'dusk' unchanged led to an advance of the whole V-pattern through a series of transient cycles; a delay of 'dusk' leaving 'dawn' unchanged, however, had little effect. These authors also showed that the shape of the activity pattern was changed with its 'amplitude', and was therefore affected by temperature and physiological states such as 'hunger'. At lower temperatures, for example, the less active flies showed relatively more of their activity in the morning peak. Brady and Crump estimated that about 80 per cent of the pattern of the field rhythm was due to the endogenous circadian clock. They suggested a complex coupling between the clock and the overt rhythm in which the shape of the bimodal rhythm was a function of its 'amplitude'. In a more recent paper, Kyorku and Brady (1994) described a bimodal activity pattern in the xerophilic tsetse species, *G. longipennis*, in which both the dawn and dusk peaks free-ran in DD with $\tau \sim 23$ hours.

6. *Circa-bi-dian rhythms (day-skipping)*

Simple unimodal free-running rhythms of behaviour, or more complex, often bimodal rhythms with an endogenous period (τ) close to 24 hours have been described above. This section deals with locomotor rhythms that occasionally adopt a longer periodicity close to 2τ (about 48 hours). Clifton (1984a, b; 1985), for example, maintained adults of the mosquito *Culiseta incidens* in continuous darkness (DD) for up to 14 weeks and measured their flight activity rhythms. Actograms were either stably circadian ($\tau < 24$ hours) or labile with changes to a longer period. Some mosquitoes adopting the longer period were observed to undergo 'day-skipping' in which overt activity was omitted on every other day so that the apparent, or overt, periodicity approached 2τ or 48 hours. Such rhythms are called circa-bi-dian, and have been described in some other organisms, including man. In *Culiseta*, the phenomenon was attributed to a complex circadian pacemaker comprising a labile evening (E) oscillator and a more stable morning (M) oscillator, circa-bi-dian rhythms appearing when the two components uncouple and ran with different periods.

C. DEVELOPMENTAL PLASTICITY OF THE CIRCADIAN PERIOD

Although the free-running period (τ) of a circadian oscillator is genetically determined (see Chapter 4), the observed value of τ may be affected by the immediate action of variables such as temperature and light intensity (sections 3, 4). Moreover, in a ground-breaking series of experiments with the cockroach *Leucophaea maderae*, Page and his associates described a developmental 'plasticity' of the circadian period whereby the light cycle experienced by the nymphs during their post-embryonic development left permanent changes to the free-running period of the adults. For example, Barrett and Page (1989) raised nymphs of *L. maderae* in

light cycles of different period (T 22, 24 or 26 hours), or in constant darkness (DD) or continuous light (LL), and then tested them, as adults, for their free-running period in darkness. Cockroaches raised as nymphs in a 22 hour cycle (LD 11:11) were shown to exhibit shorter values of τ_{DD} than those raised in a 24 hour cycle (LD 12:12). Those raised in a 26 hour cycle (LD 13:13), however, had significantly *longer* values of τ_{DD} (Fig. 2.13) and those raised as nymphs in DD had a significantly shorter value of τ_{DD} than those raised under continuous light. The observed changes in τ_{DD} were stable for at least 7 months of adult life and could not be reversed by an adult exposure to either LD 12:12 or LD 6:18. They were thus considered to be permanent, rather than merely long-lasting 'after-effects' (Chapter 6). Page and Barrett (1989) and Page (1991) also showed that nymphal exposure to abnormal light cycles had permanent effects on the *shape* of the adults' phase response curve (PRC). Those raised in LD 11:11 (T = 22 hours) showed a reduced delay portion of the PRC, whereas those raised in LD 13:13 (T = 26 hours) showed a reduced *advance* portion (see section E2).

Light cycles experienced during the post-embryonic development of *Drosophila melanogaster* also appear to have lasting effects on the adult flies' free-running period. Tomioka et al. (1997) raised larvae of *D. melanogaster*, wild type and *period* mutants, in a range of conditions including DD, LL and various 24-hour LD ratios from LD 4:20 to LD 20:4.

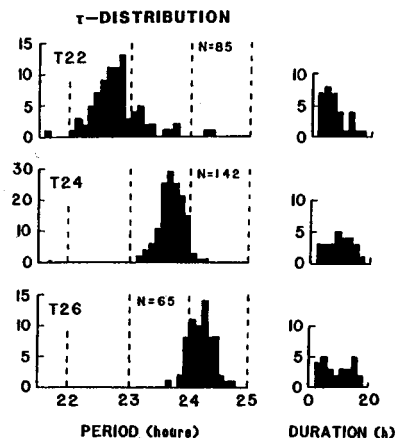


Fig. 2.13. Developmental 'plasticity' of the free-running period of *Leucophaea maderae*. Left: Distribution of periods of free-running rhythms of locomotor activity of animals raised from the time of hatching in either LD 11:11 (T = 22 hours), LD 12:12 (T = 24 hours) or LD 13:13 (T = 26 hours). Right: Distribution of values of α (duration of daily activity peak) for animals raised in the same LD cycles. (From Barrett and Page, 1989).

Locomotor activity of the adult flies was then recorded in DD free-run after an initial 7 days in the rearing regime. Wild-type (Canton-S) flies showed a shorter value of τ_{DD} after having been reared under LL, LD 20:4 and LD 16:8 than when reared under DD, and a *longer* value of τ_{DD} when reared under LD 12:12 and LD 8:16. Also, when compared with DD-reared flies, τ_{DD} for the short period mutant (*per^S*) was again shorter in LL and the longer photoperiods, but longer after being raised in LD 12:12 and LD 8:16. Adults of a long period mutant (*per^{L1}*) exhibited shorter values of τ_{DD} after all rearing regimes (LD 4:20 to LL).

Also working with *D. melanogaster*, Dowse and Ringo (1989) raised wild-type flies for several generations in the complete absence of light and then tested newly emerged adults for rhythmicity. Less than a quarter (23 per cent) of them showed wild-type activity rhythms and

half of these contained strong ultradian components. An additional 10 per cent presented weak short-period, *per*^S-like (τ 20 to 22 hours) rhythms; the remaining 68 per cent resembled either *per*^O (arrhythmic) or *per*^L (long period) genotypes. It was hypothesised that post-embryonic exposure to light may be necessary to couple a population of ultradian oscillators into a functional circadian clock. We will return to such ideas in Chapter 6.

D. EXOGENOUS AND 'MASKING' EFFECTS

At the beginning of this chapter it was stated that most daily rhythms of activity and behaviour involve a mixture of endogenous and exogenous effects, and that the 'pattern' of activity is always strongly modulated by the immediate character of the environment. Such an effect is to be seen, for example, in the light-on activity peak in the mosquito *Anopheles gambiae* which is a *direct response* to the abrupt transition from darkness to light. This 'startle' reaction disappears in DD or if a gradual increase in light intensity is substituted for the abrupt change (Jones et al., 1972).

In some species such as the locust *Schistocerca gregaria* (Odhiambo, 1966) and the stick insect *Carausius morosus* (Godden, 1973) daily activity patterns in a light-cycle appear to be largely, if not entirely, exogenous. For example, locusts transferred from LD 12:12 to DD became almost totally inactive, whereas in LL their locomotor activity became almost constant. When they were transferred to a reversed cycle of light and dark (i.e. from LD 12:12 to DL 12:12) they adopted the new activity pattern *immediately* with none of the transient cycles expected from the re-entrainment of an oscillatory system. In stick insects, locomotor and oviposition rhythms frequently disappeared after transfer to DD with activity becoming equally distributed throughout the 24 hour period. In only a few cases was a weakly persistent (i.e. circadian; τ about 24.3 hours) rhythm observed (Godden, 1973).

In probably the majority of species environmental light (for example) may produce direct, exogenous – or '*masking*' – effects on activity that are distinct from light's entraining action (see section E). Such light effects may frequently provide a form of 'fine tuning' to the rhythmic output observed in natural or 'field' rhythms. In such cases, light may elicit either 'positive' or 'negative' masking. The former occurs when light causes a direct *increase* in activity, usually in day-active species; the latter occurs when light directly *inhibits* activity, as in night-active species (see Fig. 2.1, for example). Other related phenomena, such as exogenous 'rebound effects' with temporary increases in activity after the end of the light phase may also be observed – for example, in the blow fly *Calliphora vicina* (see Fig. 2.15). Such masking effects may be distinguished from circadian entrainment by their disappearance as soon as light is removed.

It is likely that most forms of crepuscular activity contain a strong exogenous component because the period of the day at which the activity can occur is restricted by environmental factors, particularly light intensity. Tychsen and Fletcher (1971), for example, showed that mating in the Queensland fruit-fly *Dacus tryoni* occurred at an optimal light intensity of about 0.8 lux/ft². Under constant low light of this intensity mating followed a circadian rhythm which free-ran with an endogenous periodicity (τ) of about 28 hours; this endogenous component persisted for about 4 days. Conditions of constant darkness (DD) strongly depressed mating activity, however, and constant light (LL) of high intensity (900 lux/ft²) completely inhibited it. Nevertheless, 'test dusks' given to samples of flies which had been kept in high-intensity continuous light revealed an endogenous rhythm of 'readiness to mate' which persisted for two or three cycles before damping out. In the laboratory an

instantaneous step-down from high to optimal light intensity applied at the normal time of dusk was an effective stimulus to mate. Under natural photoperiods the fact that mating was suppressed by both high light intensity and by darkness restricted mating activity to a remarkably short period of 30 minutes each day at dusk when the light intensity reached its optimum (Fig. 2.14). The adaptive value of limiting mating to dusk is presumably because it synchronises the sexual behaviour of all individuals in the population and thereby increases mating efficiency. Differences in mating times between different species of *Dacus* are also known to constitute effective barriers to hybridisation.

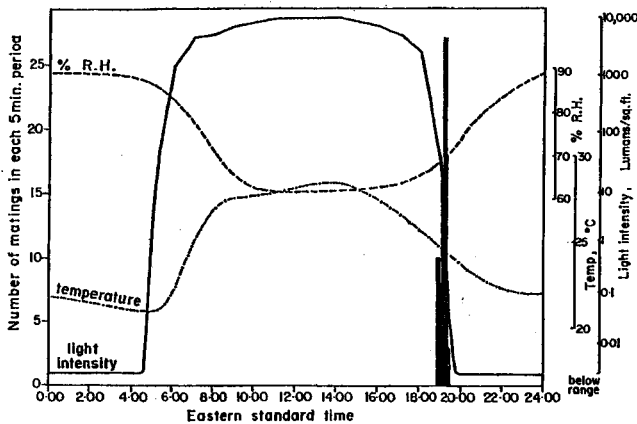


Fig. 2.14. Histogram of the number of matings of the Queensland fruit-fly *Dacus tryoni* occurring in each 5-minute period during a day under natural conditions. The changes in relative humidity, temperature and light intensity are also shown. (From Tychsen and Fletcher, 1971).

E. ENTRAINMENT BY LIGHT AND TEMPERATURE

1. Entrainment by light-cycles

When an endogenous oscillator (with period τ) is subjected to an environmental light-cycle (with period T) the period of the oscillator becomes the same as that of the *Zeitgeber*, provided that the latter is within the oscillator's range of entrainment. In natural light-cycles the oscillator therefore adopts a period which is exactly that of the solar day, namely 24 hours, and is considered to be entrained (for example, see Fig. 2.15). In its entrained steady state the overt phase of the rhythm also adopts a fixed phase-relationship (ψ) to the environmental cycle; entrainment thus constitutes both *period-control* and *phase-control*. In examining entrainment of a circadian rhythm to an environmental cycle both the period of the *Zeitgeber* (T hours) and the number of hours of light per cycle (the photoperiod) are important.

Roberts (1962) studied entrainment of the locomotor activity rhythms of *Leucophaea maderae* and *Periplaneta americana* to a variety of light-cycles in which both T and photoperiod were altered. When exposed to 24-hour cycles with a 12-hour photoperiod (LD 12:12, $T = 24$ hours) the rhythms always attained a 24-hour period to match that of the *Zeitgeber*, with activity commencing at dusk. The locomotor activity rhythm in *L. maderae*

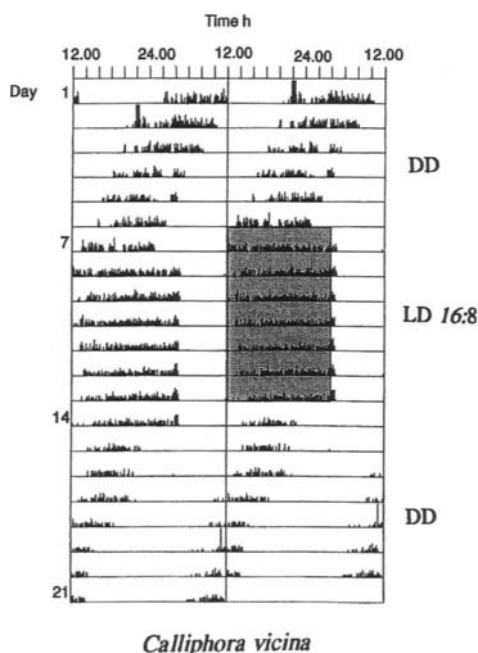


Fig. 2.15. Entrainment of the locomotor activity rhythm of a female blow fly (*Calliphora vicina*) by a light-dark cycle. The fly was initially free-running in DD, then exposed to LD 16:8 (light on in shaded box), and finally in DD. During light entrainment the fly's activity changes from $\tau < 24$ hours in DD to the period of the *Zeitgeber* (24 hours) with the activity almost entirely confined to the light portion of the cycle. On final transfer to DD locomotor activity resumes its DD free-run. (Original).

also became entrained to 24-hour cycles in which the photoperiod was other than 12 hours. When the photoperiod was long (e.g. LD 16:8 or LD 23:1) the overt phase of the rhythm and its pattern altered. With these very long light periods, as with LL, there was a pronounced secondary activity following the primary onset at dusk by about 7 or 8 hours. On return to DD this secondary peak of activity disappeared indicating its probably exogenous nature.

Roberts (1962) also demonstrated that the locomotor rhythm in *L. maderae* became entrained to a 24-hour period by some LD cycles whose periods were *sub-multiples* of 24 hours. Some individuals, for instance, entrained to cycles of LD 2:2 ($T = 4$ hours) or LD 4:4 ($T = 8$ hours), but entrainment failed with LD 1:1 ($T = 2$ hours). This phenomenon, recognised as 'frequency demultiplication', has also been recorded for the rhythm of flight activity of *Aedes taeniorhynchus* in a cycle of LD 6:6 (Nayer and Sauerman, 1971) and for the beetle *Carabus cancellatus* in LD 1:1 and LD 6:6 (Lamprecht and Weber, 1973). In the blow fly *Calliphora vicina*, Kenny and Saunders (1991) examined rhythms of locomotor activity in light cycles longer than 24 hours. Entrainment, in which flies adopted a period of 24 hours, was observed in light cycles with *Zeitgeber* periods which were *multiples* of 24 hours (e.g. LD 12:36, $T = 48$ and LD 12:60, $T = 72$) but not with those whose periods were far from multiples of 24 hours (e.g. LD 12:24, $T = 36$ and LD 12:48, $T = 60$). In the former cases, where the 12 hour light pulses 'came on' at the 'expected' phases, locomotor activity bands occurred every 24 hours, whereas in the latter cases, complex patterns were produced consistent with constant

phase adjustment of the circadian oscillator brought about by light pulses falling 'out-of-phase' every few cycles. This phenomenon may be regarded as 'frequency multiplication'.

2. Phase-shifting by light pulses, and the phase response curve (PRC)

Following the experimental protocols established by Pittendrigh (1960) for the eclosion rhythm in *Drosophila pseudoobscura* (Chapter 3), Roberts (1962) showed that single or repeated light signals applied to the rhythm of locomotor activity in *L. maderae* 'reset' the rhythm to a new phase, causing either advance phase-shifts ($+\Delta\phi$) or delay phase-shifts ($-\Delta\phi$) according to the circadian phase of the oscillator subjected to the light perturbation. Attainment of a new phase by the overt rhythm, however, was not instantaneous: the ultimate steady state was reached via a series of non-steady-state or 'transient' cycles. Characteristically the number of transients was greater in the case of phase-advances than with phase-delays.

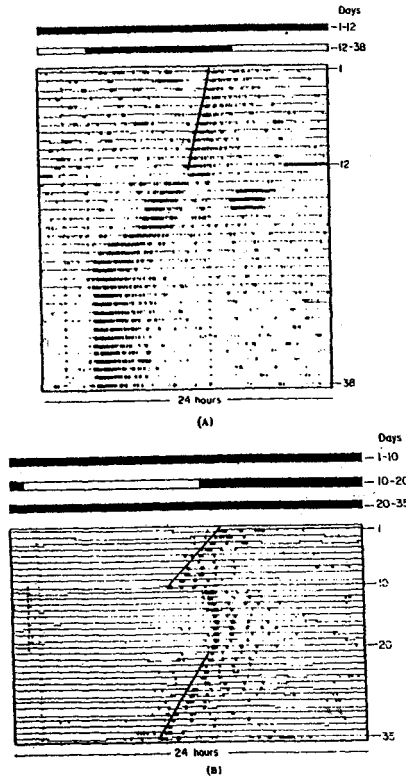


Fig. 2.16. Resetting the activity rhythm in *Leucophaea maderae* by light cycles. A – resetting with a light cycle (LD 12:12) initially out of phase with the free-running rhythm and with 'dusk' falling during the insect's subjective day. The onset of running is gradually phase advanced ($+\Delta\phi$) until entrainment is achieved after about eleven transient cycles. B - resetting with a light cycle (LD 12:12) initially out of phase and with 'dusk' falling during the insect's subjective night. The onset of running is rapidly phase delayed ($-\Delta\phi$) and the rhythm becomes entrained. Subsequent transfer to DD (on day 20) shows that the phase of the endogenous rhythm has been shifted by the light treatment. (From Roberts, 1962).

A single 12-hour light signal commencing during the middle of a cockroach's 'subjective day' caused a phase-delay of about 2 hours. A similar pulse applied during the 'subjective night' caused a 1-hour phase-advance (Roberts, 1962). The magnitude of these phase shifts was therefore considerably less than those obtained for *D. pseudoobscura* (Pittendrigh, 1960, 1965) in which light pulses of 15 minutes can induce up to 10- or 12-hour advances or delays according to the circadian time at which the pulses are given (Chapter 3). The effects of repeated out-of-phase signals were also investigated in *L. maderae*. When the insects were transferred from DD into LD 12:12 so that the first 12-hour light-pulse occurred during the insect's subjective night, the rhythm of activity phase-advanced by about 8 hours, but required about eleven transient cycles to effect the shift (Fig. 2.16A). Conversely, when the light regime started during the insect's subjective day, the rhythm phase-delayed by about 2½ hours, but required many fewer transients to reach steady-state entrainment (Fig. 2.16B). Subsequent transfer to DD demonstrated that the phase of the endogenous rhythm had indeed been shifted by the light treatment. Re-entrainment of the locomotor rhythm in *P. americana* after a reversal in the LD cycle also required a series of transient cycles before its steady-state was achieved (Harker, 1956). Although Roberts (1960, 1962) did not pursue a systematic analysis of single pulse resetting patterns in *L. maderae*, such studies, leading to phase-response curves, have subsequently been undertaken.

In order to construct a *phase-response curve* (PRC) one full circadian cycle (τ) of the organism in DD free-run is systematically perturbed by single pulses of light. Since τ varies between individuals, species and no doubt strains of species, it is necessary to normalise this period, either in terms of 'circadian hours', each being equivalent to $\tau/24$ hours, or in terms of 'angle degrees' (0 to 360°). It is customary to regard the first 12 circadian hours (Ct 00 to Ct 12) or the first 180° as 'subjective day' and the second 12 circadian hours (Ct 12 to 24) or the second 180° (180 to 360°) as 'subjective night'. For cockroaches and other nocturnal organisms, locomotor activity (α) occurs during the subjective night and 'rest' (ρ) during the subjective day. An arbitrary phase reference point must also be selected; this may be the onsets of the activity 'band', the ends of the activity 'band', or its midpoint. Since the onsets of activity are frequently the most reliable in cockroach studies, these are selected as a phase reference, although midpoints are preferred by some authors for theoretical reasons. Similarly the phase shifts generated by the light-pulses are most frequently plotted against the phase of the onset of the light-pulse, although once again midpoints are sometimes used. In cockroaches and crickets the onset of locomotor activity is frequently regarded as occurring at the start of the subjective night, or at Ct 12.

Phase-response curves have been obtained for two cockroach species, *Leucophaea maderae* (Wiedemann, 1977c) and *Nauphoeta cinerea* (Saunders and Thomson, 1977). Working with males of *N. cinerea*, and using the onset of activity as a phase reference point, Saunders and Thomson showed that single 3-hour pulses of white light ($240 \mu\text{W cm}^{-2}$) gave average phase delays ($-\Delta\phi$) of about 4 hours when the light-pulses commenced in the early subjective night (Ct 12 to Ct 18) but phase advances ($+\Delta\phi$) of up to 4 hours when starting in the late subjective night (Ct 18 to Ct 24). Phase advances were distinguished from phase delays, not only by their final steady state in relation to the activity onset before the pulse, but also on the criterion of reaching that steady state through a series of non-steady state or transient cycles which were evident for advances but not delays. A 12-hour light-pulse of the same intensity gave rise to much larger phase shifts (up to 12 hours) (Fig. 2.17) which in all but one case were devoid of such transients. The 3-hour PRC was regarded as a low 'amplitude' or 'weak' curve equivalent to Winfree's (1970a) Type I (see Chapter 3, C. 1). The

12-hour curve was regarded as an extreme form of his Type 0 or 'strong' PRC, in which the circadian oscillation is reset to a constant phase (equivalent to Ct 12) regardless of the circadian time of the onset of the light-pulse. Subsequent experiments with 6- and 9-hour light pulses have shown that the former provides a Type I curve, and the latter a curve which approaches Type 0 (Saunders, unpublished) (Fig. 2.17).

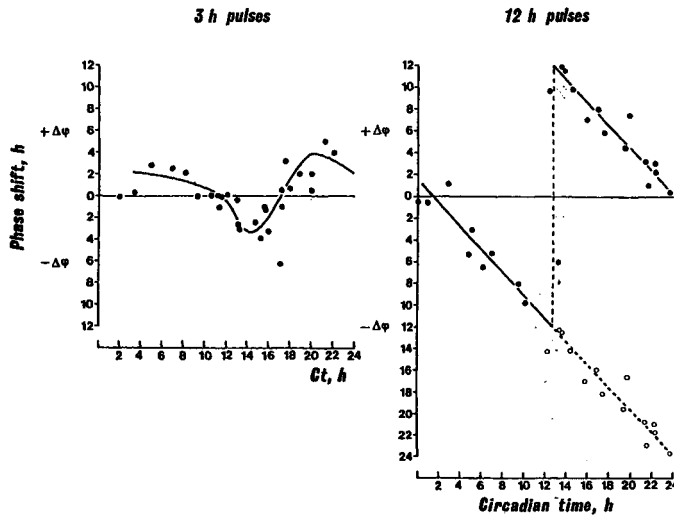


Fig. 2.17. Phase response curves (PRCs) for phase shifts of the locomotor activity rhythm in the cockroach *Nauphoeta cinerea* subjected to single pulses of white light ($240 \mu\text{W cm}^{-2}$) of 3 or 12 hours duration. $+\Delta\phi$ - advance phase shifts; $-\Delta\phi$ - delay phase shifts; Ct - circadian time scale in hours. PRC for 3 hour pulses is of Winfree's (1970a) Type 1; that for 12 hours is Type 0. (From Saunders and Thomson, 1977, and original).

Although Roberts (1962) obtained only small phase shifts in *L. maderae* with 12-hour light-pulses (2000 lux), larger phase shifts, and PRCs, have been obtained for this species by using very much higher intensity light (Wiedenmann, 1977c). Plotting phase shifts against the midpoint of the light-pulses, which were up to 80,000 lux ($32,000 \mu\text{W cm}^{-2}$), Wiedenmann obtained 'weak' or Type 1 PRCs with pulses lasting 90 or 120 angle degrees (~ 6 or 8 hours), but 'strong' or Type 0 curves for pulses lasting 180 or 205 angle degrees (~ 12 or 14 hours). Like those for *Nauphoeta* the Type 1 curves were asymmetrical with delays larger than advances (Fig. 2.18). Unlike *Nauphoeta*, however, the intensity of the light required to elicit a strong PRC was about 2 orders of magnitude greater: this presumably reflects differences in photoreceptor sensitivity. A 'critical' or 'singular' pulse, which 'abolishes' phase and leads to arrhythmicity (Winfree, 1970a; see Chapter 3, E.3), with a strength intermediate between that generating Type 1 and Type 0 curves, and commencing close to the point of phase inversion (between delays and advances) has been sought, but not found, in both *Nauphoeta* and *Leucophaea* (Wiedenmann, 1977b). Such a singularity, however, may have been located in the New Zealand weta *Hemideina thoracica* (Christensen, 1978) (Chapter 5)

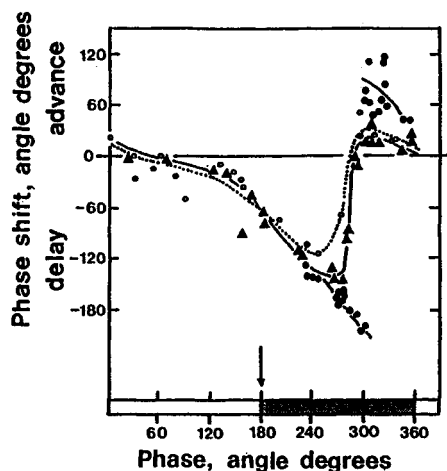


Fig. 2.18. *Leucophaea maderae*. Phase response curves for the locomotor activity rhythm with high intensity light pulses. Phase shifts (advances and delays) and phase are plotted as angle degrees; data points are plotted for mid points of the light pulses (see text). Subjective day and subjective night are indicated by white and hatched bars on the abscissa; the vertical arrow shows the onset of locomotor activity. Open circles – 50,000 lux, pulse duration 90 angle degrees (~ 6 hours); triangles – 50,000 lux, 120 angle degrees (~ 8 hours); closed circles – 80,000 lux, 180 angle degrees (~ 12 hours). (From Wiedenmann, 1977).

Phase response curves have also been constructed for wild type and *period* mutants (see Chapter 4) of *Drosophila melanogaster* (Saunders et al., 1994). Wild type (Canton-S), short-period (*per^S*) and long period (*per^{L2}*) flies were allowed to free-run in DD and then exposed, at all circadian times, to 1, 6 or 10 hour pulses of white light. PRCs so obtained showed a change from a low 'amplitude' Type 1 (Winfree, 1970a) with 1 hour pulses, to high 'amplitude' Type 0 for 6 and 10 hour pulses. These PRCs were later simulated using a feedback control systems model for the circadian clock (see Chapter 7)(Lewis et al., 1997). Other light pulse PRCs have been described for a number of species, including the blow flies *Lucilia cuprina* (Smith, 1983) and *Calliphora vicina* (Cymborowski et al., 1993), and for the cricket *Gryllus bimaculatus* (Okada et al., 1991).

The phase-response curve provides the means for calculating the approach to steady-state entrainment to trains of pulses (of period T hours). In particular, it enables calculation of the *primary range of entrainment*, and the entrained *phase angle* (ψ -value) between the light-pulses and the rhythm. For example, in the case of *N. cinerea* exposed to trains of 3-hour pulses, the locomotor rhythm may be expected to entrain to T -values encompassed by the maximum delays and advances, about $T = 20$ hours to $T = 28$ hours in this case. For longer or brighter pulses, the potential range would be greater. The effect of T on phase angle (ψ) is exemplified by data with *H. thoracica* (Christensen, 1978). The range of entrainment for this species exposed to 8-hour pulses of light (< 100 lux) was about $T = 21$ to $T = 31$ hours. When entrained to $T = 23$ (LD 8:15) the 'band' of locomotor activity occurred towards the end of the dark period; when entrained to $T = 25$ (LD 8:17) it occurred towards the beginning. The phase angle (ψ) between activity and the entraining pulses was clearly a function of T , as in rodents (Pittendrigh and Daan, 1976) and the fruit-fly *Drosophila pseudoobscura* (Chapter 3, C.2).

Control of phase angle (ψ) was also examined in the blow fly *Protophormia terraenovae* by Aschoff and von Saint Paul (1990). Adult flies were maintained in small

running wheels and exposed to light cycles (L = 400 lux, ‘D’ = 2 lux) each of which, although of a different period, contained the same ratio of light to darkness (i.e. LD 11:11, T = 22; LD 12:12, T = 24; LD 13:13, T = 26 and LD 14:14, T = 28 hours). Phase angles (ψ) between activity onsets and ‘light-on’, and activity end and ‘light-off’ were determined. Flies were found to entrain to the range from T = 24 to T = 28 hours; within this range of entrainment, ψ -values changed from phase lagging ($-\psi$) in T = 24 to phase leading ($+\psi$) in T = 28 hours.

The range of entrainment of the circadian pacemaker to the light cycle was determined for the blow fly *C. vicina* in a range of light cycles from LD 12:8 (T = 20 hours) to LD 12:18 (T = 30 hours) (Kenny and Saunders, 1991), these being close to the limits of entrainment using light pulses of this duration. Under LD 12:8 some of the flies failed to entrain and free-ran through the light cycle; others which did entrain adjusted the period of their rhythm to that of the *Zeitgeber* (T = 20 hours) and attained the expected phase-lag to the light. Under LD 12:18, although some failed to entrain, those that did so adjusted the period of their rhythm to T = 30, but showed characteristic phase-leading behaviour (Fig. 2.19).

Phase-setting and entrainment of flight rhythms by light-pulses and cycles have been investigated in the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. After an advance in the light-cycle achieved by shortening either one light or dark period, Jones et al. (1967) showed that the rhythm of flight activity in *A. gambiae* required several transient cycles before reaching its final steady state. In *A. aegypti*, a reversal of the LD 12:12 light-cycle by prolonging either one dark or one light period to 24 hours caused a resetting of the activity rhythm by a change in the time of light-off (Taylor and Jones, 1969).

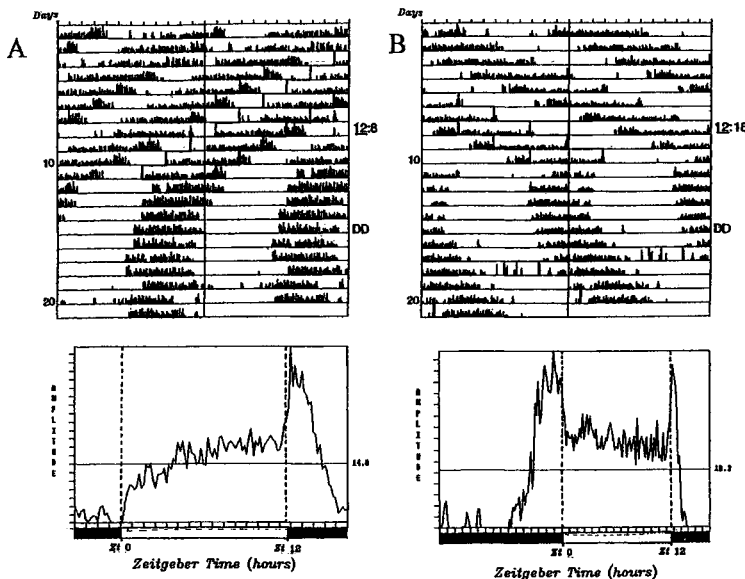


Fig. 2.19. *Calliphora vicina*. Entrainment of the locomotor activity rhythm to non-24 hour light-dark cycles (at 20°C). A – to LD 12:8 (T = 20 hours), then continuous darkness; B – to LD 12:18 (T = 30 hours), then continuous darkness. In T = 20 the rhythm entrains with a pronounced phase-lag to the *Zeitgeber*; in T = 30 it also entrains, but with a pronounced phase-lead. The lower panels show average activity profiles to show phase-lagging (A) and phase-leading (B) behaviour. (From Kenny and Saunders, 1991).

Jones et al. (1972a) have also provided a phase-response curve for *A. gambiae*. Mosquitoes were raised at 25°C and LD 12:12 and individual females were then subjected to 1-hour pulses of white light (70 lux) at different times in the first circadian cycle following a transfer to DD. The steady-state phases of the subsequent activity peaks were then measured at the end of the second cycle when transients had subsided. The phase-response curve so produced showed that the mean $\Delta\phi$ obtained was about 2 hours. Pulses applied late in the subjective night caused phase advances ($+\Delta\phi$); those in the subjective day or early in the subjective night caused phase delays ($-\Delta\phi$). The phase-response curve also showed the abrupt change between $-\Delta\phi$ and $+\Delta\phi$, which is also seen in *D. pseudoobscura* (Chapter 3) and all other species so examined (Aschoff, 1965). In *A. gambiae*, the magnitude of $\Delta\phi$ was found to be a function of signal energy. Five minutes at 70 lux was insufficient; with a 1-hour pulse $\Delta\phi$ increased with pulses up to 500 lux, but was insignificant below 10 lux.

The way in which phase response curves are used to calculate the approach to steady-state entrainment and the resulting phase angles (ψ -values) will be described in Chapter 3 with reference to the pupal eclosion rhythm of *Drosophila pseudoobscura*. A full account of PRCs and their uses may also be found in the excellent review by Johnson (1992)

3. Spectral sensitivity of photic entrainment

Sokolove (1975) described a variety of red-light effects on the cricket *Teleogryllus commodus*. Cycles of red light and darkness were found to entrain both the locomotor and the stridulatory rhythms. Continuous exposure to red light (>600 nm), on the other hand, caused τ to lengthen from about 23.8 to about 25.3 hours. After a subsequent transfer to DD, τ shortened, but took nearly 3 weeks to regain its original period. This observation is an example of a class of phenomena called 'after-effects' (Pittendrigh, 1960), in this case an after-effect of previous exposure to red light; such phenomena will be considered further in Chapter 6. Wiedenmann (1978) observed similar period-lengthening effects of red light for males of *Leucophaea maderae*. In some specimens of this cockroach cycles of red light and darkness entrained the rhythm of locomotor activity; in others they failed to 'capture' the free-running rhythm, resulting in so-called 'relative co-ordination'; still others merely free-ran through the red-light cycles with an unchanged period. These differences presumably reflect differences in photoreceptor sensitivity.

A systematic study of the spectral sensitivity of light-mediated effects (phase shifts and ultimate entrainment) was carried out by Mote and Black (1981) using the locomotor activity rhythm of the cockroach *Periplaneta americana*. In this insect there are two receptor types in the relevant photoreceptor (the compound eye; see Chapter 8), one maximally sensitive to UV, the other responding best to green. The entrainment action spectrum for *P. americana* showed maximum sensitivity near 495 nm, presumably involving the green-sensitive receptors (Fig. 2.20). More recently, Leppla et al. (1989) reported that the locomotor activity rhythm of the cockroach *Bletella germanica* entrained to cycles of LD 12:12 in which the light component was either white (400 to 1100 nm) or monochromatic (peaks at 365 or 495 nm) with a bimodal nocturnality which subsequently free-ran after transfer to DD.

4. Entrainment by temperature cycles

In the natural environment the daily cycle of light intensity is associated with a concomitant cycle of temperature, the normal phase relationship being when dawn falls

somewhere near the low point of the temperature cycle. It is not surprising, therefore, that a daily temperature cycle, as well as a light-cycle, can act as a *Zeitgeber* and entrain circadian rhythms. Entrainment by temperature cycles will be illustrated by reference to locomotor rhythmicity in cockroaches and flies.

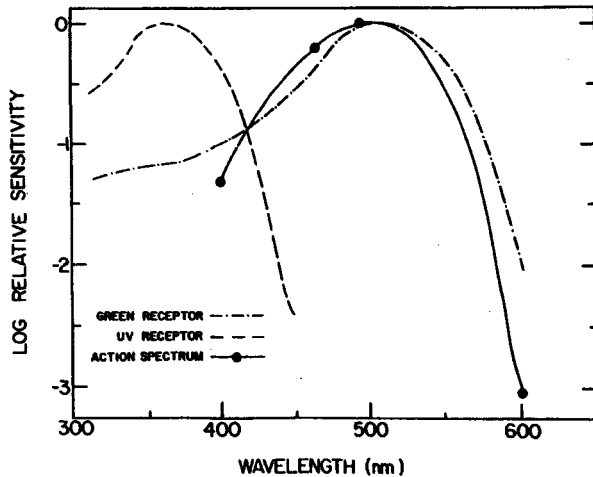


Fig. 2.20. Action spectrum for entrainment (closed circles) in the cockroach *Periplaneta americana* compared to the spectral sensitivities of the two classes of photoreceptor (green and UV) in the compound eye. (From Mote and Black, 1981).

Roberts (1962) demonstrated that a 24-hour temperature cycle could entrain the locomotor rhythm in cockroaches (*Leucophaea maderae* and *Periplaneta americana*) held in otherwise constant conditions, despite the fact that earlier reports by Cloudsley-Thompson (1953) and Harker (1956, 1958) were conflicting or contradictory on this point. Figure 2.21, for example, shows an individual of *L. maderae* maintained throughout in DD. For the first 17 days the temperature was held at a constant 25°C and the locomotor activity rhythm free-ran with an endogenous periodicity less than 24 hours. From day 17 to day 44 the insect was subjected to a 24-hour sinusoidal temperature cycle fluctuating between 22° and 27°C; the rhythm assumed the 24-hour period of the *Zeitgeber* with the onset of activity close to the high point of the cycle. After day 44 the temperature cycle was discontinued and the rhythm again free-ran.

Just as a temperature cycle can entrain a circadian rhythm in a similar fashion to a light-cycle, a temperature *pulse* will cause a phase-shift in a manner comparable to a light-pulse. For cockroaches (*P. americana*), this was first demonstrated by Bünning (1959): phase-shifts occurred when the temperature was lowered for an interval of 12 hours or less, the magnitude of the phase-shift depending on the timing of the temperature treatment. Roberts (1962) showed that a 12-hour low-temperature pulse (7° or 12°C) applied to *L. maderae*, otherwise maintained in DD at 25°C, would also effect an alteration in phase. Wiedenmann (1978) then described phase-response curves for low (1°C) and high (39°C) temperature pulses applied to males of *Leucophaea maderae* free-running in DD. Such pulses always produced phase delays. For low-temperature pulses of 120 angle degrees (~8 hours), maximum delays of about 140°

(~9 hours) were obtained when the pulses were placed towards the end of the subjective day; minimum delays of about 60° (~4 hours) occurred at the end of the subjective night. High-temperature pulses were less effective, with delays never exceeding about 18° or 1 to 2 hours.

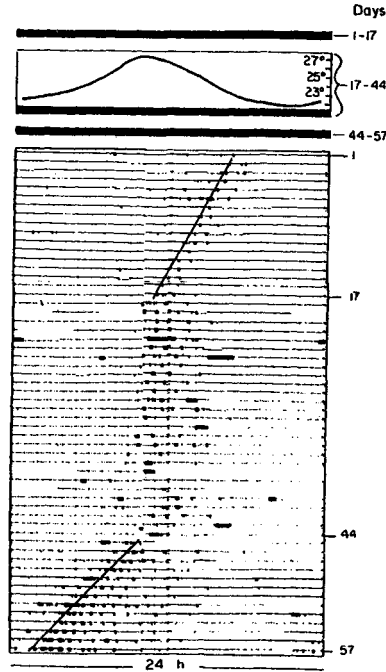


Fig. 2.21. Entrainment of the locomotor rhythm in *Leucophaea maderae* to a 24 hour sinusoidal temperature cycle (22 to 27°C) in constant darkness. (From Roberts, 1962).

Since both light and temperature cycles can act as *Zeitgeber* in entraining the rhythm of locomotor activity, interesting situations occur when the phase angle between the two environmental driving cycles is altered (Roberts, unpublished, cited in Pittendrigh, 1960). In the natural environment, as already mentioned, the coolest part of the temperature cycle occurs close to dawn whereas the warmest part occurs late in the light period. Under this situation, locomotor activity begins soon after dark. However, as the phase angle between sunset and the high point of the temperature cycle was experimentally widened, the onset of activity moved steadily into the subjective night until a discrete phase-jump occurred and the onset of activity jumped by about 12 hours to the next dusk. It seems that at least 180° of the 360° of conceivable phase relative to the light-cycle constitutes a zone of 'forbidden' phase relations, and indicates that the light-cycle is a 'stronger' *Zeitgeber* than temperature. A similar experiment with the rhythm of adult eclosion in *Drosophila pseudoobscura* will be described in Chapter 3.

Working with flight and locomotor activity in the mosquito *Culex pipiens pallens*, Chiba et al. (1993) demonstrated entrainment, in DD, to a daily temperature cycle of 16 hours at 28° and 8 hours at 23°C . Other studies on flies include Tomioka et al. (1998) who showed

that temperature cycles could entrain *Drosophila melanogaster*, wild type and *period* mutants, and Saunders and Hong (2000) with the blow fly *Calliphora vicina*. In *D. melanogaster*, a temperature cycle of 12 hours at 25° and 12 hours at 30° caused arrhythmic flies (*per*⁰) to be active in the thermophase (30°) in DD, but in the cryophase (25°) in LL. This rhythmicity was considered to be 'forced' or exogenous.

Temperature cycles comprise a train of temperature *pulses*, and pulses are defined by temperature *steps*, both up and down, in much the same way as light cycles are defined by light pulses and steps. However, the use of temperature to study entrainment has the advantage that temperature steps-up or steps-down may be studied separately, something which cannot be done with light because 'light-on' automatically leads to a period of higher light intensity, which frequently leads to arrhythmicity (section B3) in which phase has no clear meaning.

In a study of the phase shifting effects of temperature steps in *C. vicina*, Saunders and Hong (2000) showed that both steps-up (20 to 25°C) and steps-down (20 to 15°C) caused stable phase shifts of the locomotor rhythm, in DD, giving rise to temperature step phase response curves (PRCs) with both phase advances and delays. Phase advances ($+\Delta\phi$), however, were dominant for steps-up, and phase delays ($-\Delta\phi$) were dominant for steps-down; the two PRCs (Fig. 2.22) were almost 'mirror images' of each other. Following protocols introduced by Zimmerman et al. (1968) for the rhythm of pupal eclosion in *Drosophila pseudoobscura* (see Chapter 3), the temperature steps-up and steps-down PRCs for *C. vicina* were used to compute a theoretical PRC for a 6 hour low temperature *pulse*, and from this to predict the steady state phase relationship (ψ) of locomotor activity to a train of such pulses making up a temperature *cycle* of 18 hours at 20° and 6 hours at 15°C. In the computed steady state, locomotor activity was predicted to occur toward the end of the daily thermophase, appropriate for a diurnal insect in the natural environment.

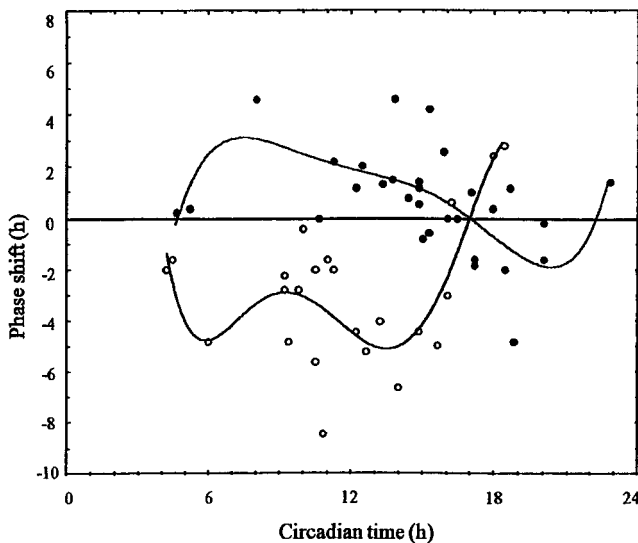


Fig. 2.22. *Calliphora vicina*. Phase response curves for 5°C temperature steps-up (closed circles; 20 to 25°C) and 5°C temperature steps-down (open circles; 20 to 15°C). Both curves show advance and delay portions, and are approximately mirror images of each other. (From Saunders and Hong, 2000).

5. Entrainment by other non-photic Zeitgeber

The daily light-dark cycle is undoubtedly the major environmental synchroniser or *Zeitgeber*, with the temperature cycle (a non-photic *Zeitgeber*) coming up second in importance. Several other non-photic *Zeitgeber*, however, have been described in recent years.

Working with colonies of the honey bee (*Apis mellifera*) maintained under continuous light (LL) at 25°C, Frisch and Aschoff (1987) presented food (1.5 M sugar solution) for 2 hours daily, for up to 2 weeks before returning the bees to a regimen of continuous sugar feeding. Colonies showed free-running activity during continuous feeding (τ 22.6 to 24.8 hours). When they were subjected to feeding cycles with sugar offered for 2 hours every 22, 23, 24 or 25 hours, however, most bees entrained to these cycles with a stable ψ -value between the onset of activity and the onset of food availability, indicating that the feeding cycle was acting as an effective *Zeitgeber* in the bees' complex behaviour. Also with honey bees, Southwick and Moritz (1987) showed that worker bees co-ordinated their individual metabolic rhythms (O_2 consumption) into a collective rhythm. The 'social' *Zeitgeber* operating here was thought to be physical interaction between the workers.

Engelmann et al. (1996) showed that the locomotor activity rhythm of adult *Musca domestica* could be affected by square wave 10 Hz cycles in the electric field. Continuous application of such a field tended to slow down the clock of shorter period flies, whereas that of longer period flies was either not affected or was accelerated. When applied for 12 hours per day, the electric field cycle led to synchronisation in 30 to 40 per cent of flies; it is probably, therefore, a weak *Zeitgeber* in its own right. Earlier investigations by Dowse (1982) had indicated that an electric field caused phase shifts in *Drosophila melanogaster*.

Finally, there is a possibility that internal, physiological factors may act as *Zeitgeber* by feeding forward (or back) onto overt rhythmicity such as locomotion. Such a possibility was indicated by Page (1987) who showed that serotonin (5-HT), a putative insect neurotransmitter, caused consistent phase shifts of the locomotor activity rhythm of *Leucophaea maderae*. When 4 μ l of a 0.05 M solution of serotonin was injected every 15 minutes for a 6 hour period, such treatment in the early subjective night (Ct 14) caused phase delays ($-\Delta\phi$) of about 4 hours, whereas a similar treatment in the late subjective night had little or no effect. The phase response curve (PRC) for serotonin administration (Fig. 2.23A), therefore, was rather different to that for light pulses which cause $-\Delta\phi$ in the late subjective day and early subjective night, but phase advances ($+\Delta\phi$) in the late subjective night. The physiological significance of the observation that serotonin 'pulses' may shift phase was considered uncertain since rather high concentrations were required to pass the insects' 'blood-brain barrier'. The possibility that serotonin functioned as a neurotransmitter in the light entrainment pathway (compound eyes to optic lobes; see Chapter 8) was rejected because of the profound differences between the PRCs for serotonin and light.

In a later study with the blow fly *Calliphora vicina*, Cymborowski (1998) showed that injection of p-chlorophenylalanine (a potent inhibitor of 5-HT synthesis) into the haemolymph of flies maintained in continuous 'bright' light (0.07 W m^{-2}) caused the flies to change from arrhythmicity to a free-running rhythm with a periodicity characteristic for constant 'dim' light (see Section B, 3, a). After flies kept in 'dim' LL were injected into the brain with 5-HT, activity became hyperactive and arrhythmic as if in constant 'bright' light. These data suggested a potential role for serotonin as a mediator of circadian changes in the photoreceptive pathway of *C. vicina*, including perhaps the extraretinal system known to be important in this insect (see Chapter 8).

Daily rhythms of steroid moulting hormones, or ecdysteroids, have been observed in the haemolymph of several insects including the blood-sucking bug *Rhodnius prolixus* (Vafopoulou and Steel, 1989, 1992; see Chapter 5), raising the possibility that such rhythms may act as some sort of internal, physiological *Zeitgeber*. Cymborowski et al (1993) used topical applications of a non-steroidal ecdysteroid mimic (RH 5849), applied to the thorax of blow flies (*Calliphora vicina*) free-running in DD, to effect phase shifts. They observed phase advances ($+\Delta\phi$) of up to 2 hours in the late subjective day and early subjective night, but phase delays ($-\Delta\phi$) of about 1½ hours when the hormone mimic was applied in the late subjective night and early subjective day. The PRC so generated by RH 5849 applications (Fig. 2.23B), therefore, was the opposite – but not an exact ‘mirror image’ – of that for 1 hour light pulses. These data indicate the possibility that fluctuations of naturally occurring ecdysteroids may act as an endogenous synchroniser within the insect’s circadian system.

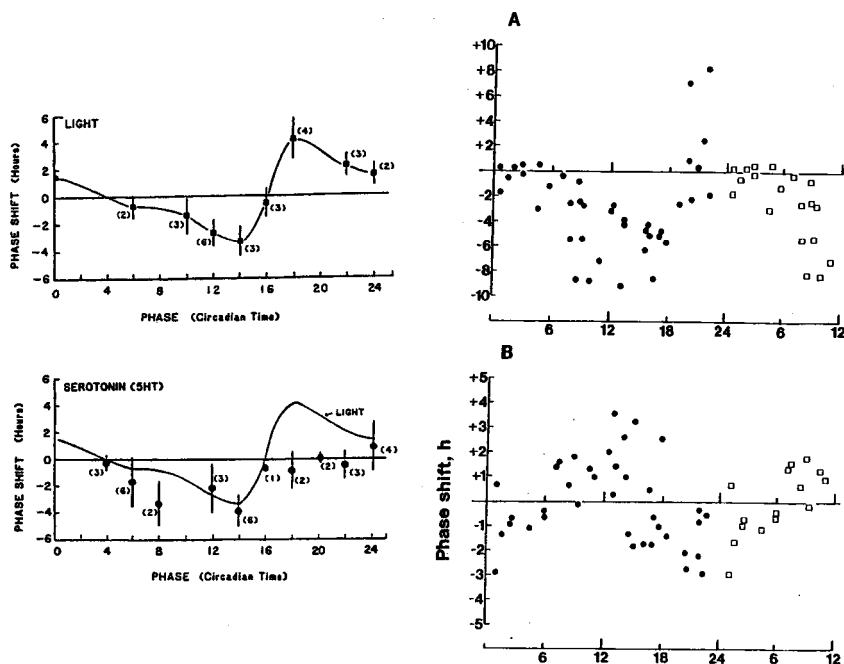


Fig. 2.23. Phase response curves for non-photic *Zeitgeber*. Left – *Leucophaea maderae*: PRC for 6 hour infusions with the neurotransmitter serotonin (lower panel) compared with that for 6 hour pulses of light. (From Page, 1987). Right – *Calliphora vicina*: PRC for topical applications of the non-steroidal ecdysteroid agonist RH5849 (lower panel) compared with that for 1 hour pulses of light. (From Cymborowski et al., 1993).

F. CLOCK 'COMPLEXITY'

Throughout this chapter the basic properties and characteristics of circadian rhythms in individual insects have been outlined as though the endogenous 'driving' mechanism was a simple (single) circadian oscillation. This simple approach will be continued in Chapter 3 ("Circadian Rhythms of Activity in Populations of Insects") even though these rhythms require mixed-age *populations* of oscillators for their overt expression. In both of these introductory chapters, however, there are numerous hints that overt rhythmicity is driven by a much more complex system of interacting circadian (and perhaps also ultradian) oscillators. This theme will be developed further in Chapter 4, in which the circadian phenomenon is presented in molecular terms as a cell-autonomous feed-back system; in Chapter 5 where diverse circadian oscillations will be described in different tissues, organs and physiological systems; and in Chapter 6 where the concept of a multioscillator, and multicellular, circadian *system* will be developed.

ANNOTATED SUMMARY

1. Most activity rhythms in individual insects have a strong endogenous component, although they may be, to a variable extent, modulated by the direct effects of the environment.
2. The endogenous nature of circadian rhythms is revealed when the insects are transferred from a light/dark cycle into continuous darkness (DD) or continuous light (LL), when the rhythm will 'free-run' and present its natural periodicity (τ) provided that temperature cycles and other potential *Zeitgeber* are carefully controlled.
3. The endogenous circadian periodicity (τ) is rarely exactly 24 hours, and normally varies (between species or between individuals of the same species) from about 22 to 27 hours. In individual insects there are often spontaneous changes of τ .
4. In many vertebrate species τ in LL is longer than in DD and lengthens as the light intensity increases if the animal is nocturnal, but is shorter or shortens with intensity if the animal is diurnal. This relationship - called 'Aschoff's rule' - is applicable to some insects, but not to all. In *Hemideina thoracica*, the increase of τ on transfer from DD to LL is greater at lower temperatures.
5. The period of the circadian oscillator in free-run is temperature-compensated, frequently showing a Q_{10} of little more than 1. (In the cockroach, *Leucophaea maderae*, for example, it is 1.04.) This remarkable biological feature is an absolute functional prerequisite for a 'clock'.
6. The period of the free-running circadian oscillator is sometimes lengthened by additions of heavy water (D_2O) or lithium ions (Li^+) to the drinking water, but is otherwise remarkably stable in the face of many chemical and physical perturbations.
7. Although the realisable *range* of τ values is genetically determined (see Chapter 4), environmental lighting conditions (i.e. DD, LL, or LD cycles with a period which differs from 24 hours) during post-embryonic development may bring about permanent changes to the expression of circadian rhythms in the adult.
8. When the free-running oscillator (with a period τ) is subjected to an environmental light cycle (with a period T), the former adopts the period of the latter (i.e. $\tau = T$), or is entrained by it, undergoing phase shifts, usually with one or more transient cycles.
9. Systematic perturbations with a single light pulse at all phases (circadian times) of the driving oscillator provide a phase-response curve (PRC) with delay phase shifts ($-\Delta\phi$) in

- the early subjective night and advance phase shifts ($+\Delta\phi$) in the late subjective night. For behavioural rhythms in individual insects, PRCs are most frequently of the 'weak' Type 1 (see Chapter 3), but may become 'strong' Type 0 with very bright or very long light-pulses.
10. The endogenous oscillator will also entrain to light-cycles whose periods (T) are different from 24 hours, provided that they are within the oscillator's primary range of entrainment. The oscillator will also entrain to light cycles whose periods (T) are sub-multiples or multiples of 24 hours, in which cases the entrained rhythms express a period of exactly 24 hours.
 11. Insect circadian rhythms will also entrain to non-photic *Zeitgeber* including cycles of temperature, food availability, social interactions, and some physiological or pharmacological agents.

CHAPTER 3

CIRCADIAN RHYTHMS OF ACTIVITY IN POPULATIONS OF INSECTS

The little, meagre, shrivelled, hopping, though loud and troublesome insects of the hour.

Edmund Burke

CONTENTS

Introduction	44
A. <i>General Properties of Population Rhythms</i>	44
1. Pupal eclosion rhythms	44
2. Oviposition rhythms	51
3. Egg-hatching rhythms	53
4. Larval rhythms	55
5. Pupation rhythms	57
B. <i>Population Rhythms as 'Gating' Phenomena</i>	58
C. <i>Entrainment of Population Rhythms by Light</i>	62
1. The phase-response curve	63
2. Entrainment by single recurrent light-pulses	70
3. Entrainment by 'skeleton' photoperiods	74
D. <i>Effects of Continuous Light and Extended Light Periods</i>	80
E. <i>The 'Singularity Point'</i>	84
F. <i>Effects of Low Oxygen Tension and Cold Torpor</i>	87
G. <i>Effects of Temperature Pulses and Cycles</i>	88
1. Entrainment	88
2. Is there a temperature-sensitive event between photoreceptor and clock?	92
3. Temperature cycles as <i>Zeitgeber</i> in the absence of light sensitivity	93
H. <i>Spectral Sensitivity and Intensity Effects</i>	94
I. <i>Some Genetic Aspects of Population Rhythms</i>	96
1. Latitudinal clines in clock parameters	96
2. Selection for phase angle: 'early' and 'late'	99
3. Heavily damped rhythms and arrhythmicity	100
4. Isolation of clock mutants	100
Annotated Summary	100

INTRODUCTION

ALTHOUGH many aspects of development or behaviour occur but once in the life-cycle of an individual insect, their timing may be controlled by an on-going circadian oscillation in such a way that they occur at a particular time of the day or the night. In such cases a clear rhythm of activity is apparent only in a population of mixed developmental ages.

Probably the most spectacular of these rhythms is the emergence of adult insects from their pupae or puparia. In *Drosophila* spp., for example, pupal eclosion occurs during the hours close to dawn (Bünning, 1935; Kalmus, 1935; Pittendrigh, 1954; Brett, 1955). It also occurs close to dawn in the yellow dung fly *Scopeuma stercoraria* (Lewis and Bletchley, 1943), the Queensland fruit-fly *Dacus tryoni* (Myers, 1952; Bateman, 1955), and in the moths *Pectinophora gossypiella* (Pittendrigh and Minis, 1964) and *Heliothis zea* (Callahan, 1958). In the flour moth *Anagasta kühniella*, however, eclosion occurs in the late afternoon and early evening (Bremer, 1926; Scott, 1936; Moriarty, 1959), and in some Chironomids it occurs at night (Palmen, 1955). In still other insects such as the mosquito *Aedes aegypti* pupal eclosion is not a rhythmic event (Haddow et al., 1959).

Other events which are clock-controlled include egg hatching in *P. gossypiella* (Minis and Pittendrigh, 1968), pupation in *Aedes* spp. (Nielsen and Haeger, 1954; McClelland and Green, 1970), and puparium-formation in the seaweed fly *Coelopa frigida* (Remmert, 1961) and the fruit-fly *Drosophila victoria* (Rensing and Hardeland, 1967; Pittendrigh and Skopik, 1970). Remmert (1962) gives an account of many of these rhythms.

Rhythms of oviposition have been extensively studied in the yellow-fever mosquito *Aedes aegypti* (Haddow and Gillett, 1957) and in the pink boll worm moth *Pectinophora gossypiella* (Pittendrigh and Minis, 1964; Minis, 1965). Strictly speaking, since each female insect deposits many eggs on a number of occasions, these rhythms are not comparable to those of eclosion or pupation. Nevertheless, in *A. aegypti* a single blood meal leads to a single batch of eggs (= gonotrophic concordance), and each female lays her eggs in one or, in the most, two consecutive daily peaks (Gillett, 1962). No further eggs are then developed until the next bloodmeal is taken. For these reasons a rhythm is clearly expressed only in a population of mixed developmental ages; hence the inclusion of oviposition rhythms in this chapter.

The best-known 'population' rhythm is undoubtedly that of pupal eclosion in *Drosophila* spp. The timing of this event attracted several early workers such as Kalmus (1935) and Bünning (1935), and was later studied extensively by C. S. Pittendrigh (1954, 1960, 1965, 1966) and his associates at Princeton and Stanford Universities. Much of what we know about any circadian system arises from Pittendrigh's work, and this example will form the basis of the present chapter.

A. GENERAL PROPERTIES OF POPULATION RHYTHMS

1. Pupal eclosion rhythms

Mixed-age populations of *Drosophila pseudoobscura* raised in alternating cycles of light and dark emerge as adults in a well-defined rhythm with peaks close to dawn. In its entrained steady state the rhythm assumes a definite phase-relationship to the driving light-cycle (Pittendrigh, 1965), the positions of the peaks depending on the length of the photoperiod. With very short photoperiods (less than 6 to 7 hours), for example, the eclosion peaks lie before dawn, whereas with longer photoperiods they occur after dawn. In LD 12:12 the median of the eclosion peak occurs about 2 to 3 hours after the onset of light (Fig. 3. 1).

This relationship is not linear, however: with photoperiods in excess of 18 hours there is a distinct change of phase and in very long photoperiods, or in continuous light of sufficiently high intensity, the rhythm becomes inapparent.

Populations raised from the egg stage in DD and constant temperature show an arrhythmic pattern, but transfer to LD at any stage of larval or intra-pupal development will result in a rhythm being generated. Bünning (1935) showed that this could also take place after cultivation in constant dim light for fifteen generations. A rhythmic emergence of adult flies can also be generated by transferring populations from LD into DD, from LL into DD, or by means of a single unrepeatable light signal - even as short as 1/2000 second - applied to an otherwise DD culture. The rhythm so produced 'free-runs' in the further absence of temporal cues and reveals its natural periodicity (τ) which, in *D. pseudoobscura*, is very close to 24 hours. If a culture is transferred from DD to LL with an intensity of 0.3 to 3000 lux the rhythm so initiated damps out after three to four cycles (Chandrashekar and Loher, 1969a). The period (τ) of the oscillation in LL is less than 24 hours (Bruce and Pittendrigh, 1957; Chandrashekar and Loher, 1969a).

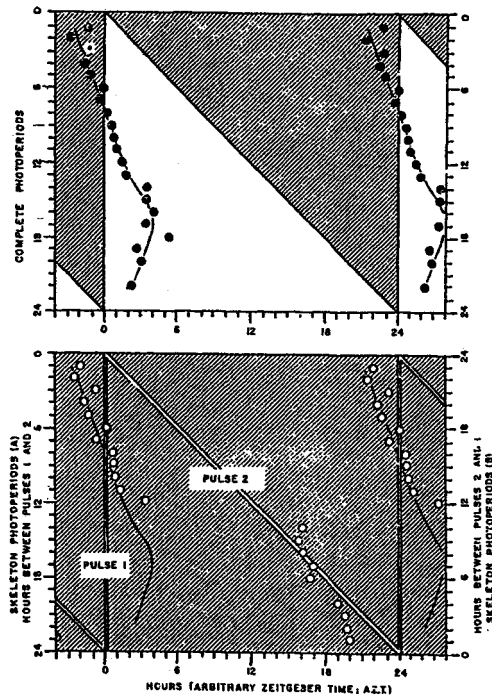


Fig. 3.1. The phase of the *Drosophila pseudoobscura* eclosion rhythm as a function of photoperiod. The plotted points are the medians of the steady state distributions of eclosion. Upper panel: in 'complete' photoperiods; lower panel: in skeleton photoperiods formed from two 15 minute light pulses per cycle (From Pittendrigh, 1965).

One of the important fundamental properties of endogenous circadian rhythms is that the period of the oscillation is temperature-compensated in the absence of temporal cues (Pittendrigh, 1954; Bruce, 1960). In *D. pseudoobscura*, for example, Pittendrigh (1954)

demonstrated that temperature effects on period were very slight indeed. Three separate cultures raised in LD 12:12 and at 16°, 21° and 26°C were transferred to DD whereupon the rhythms persisted for a further four or five cycles until all the flies had emerged. However, the period of the rhythm was not absolutely 'temperature-independent': at 26° it was about 24 hours, whereas at 16° it had slowed to about 24.5 hours (Fig. 3.2). Zimmerman et al. (1968) have since shown that the period was 24.7 hours at 10°, 24.0 hours at 20°, and 23.7 hours at 28 °C. This slight positive dependence of τ on temperature ($Q_{10} = 1.02$) is important because it demonstrates that the rhythm is not being entrained by an uncontrolled aspect of the 24-hour environmental cycle (in which case the Q_{10} would have been 1.0), and amply demonstrates the endogenous nature of the rhythm. A near-24-hour periodicity in DD is of clear selective value for *D. pseudoobscura* and other flies with subterranean pupae since it enables the species to 'remember' the time of the last-seen dawn throughout intrapuparial development and still emerge at the 'right' time.

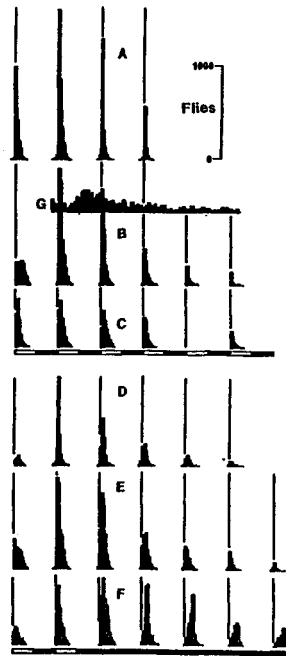


Fig. 3.2. The rhythm of pupal eclosion in *D. pseudoobscura*. A - 26°C, B - 21°C and C - 16°C are from cultures maintained throughout their development under LD conditions, as indicated by the light regime represented under C. D - 26°C, E - 21°C and F - 16°C are from cultures transferred, without temperature change, from LD to DD, as indicated by the light regime bar under F. G shows the aperiodic distribution of eclosion that is seen in cultures raised in DD conditions. (From Pittendrigh, 1954).

Although the period of the rhythm is almost unaffected by temperature, certain other aspects are markedly temperature-sensitive. For example, since the rate of development varies with temperature, the population raised at 26°C utilised a smaller number of peaks than that at 16°C, but produced a larger proportion of the total population in each. In other words, although the period of the rhythm was temperature-compensated, its 'amplitude' was not. The overt

phase of the eclosion peaks also occurred later, relative to dawn, as the temperature was lowered. A third effect of temperature was seen when a marked temperature change occurred at the same time as the transfer from LD to DD. Kalmus (1940) claimed that a rise in temperature at this point caused a shortening of the period, whereas a drop in temperature caused the period to be lengthened. On the basis of these observations he suggested that the endogenous rhythm controlling adult eclosion in *Drosophila* was temperature-dependent. Pittendrigh (1954), however, was able to demonstrate that it was only the first peak after the temperature change that was so affected, a fact apparently overlooked by Kalmus. Immediately after the initial delay or acceleration the system reverted to an essentially 24-hour periodicity only a little out-of-phase with that obtaining before the temperature shock. In 1954 Pittendrigh attributed the advance or delay of the first peak to a temperature-sensitive 'terminal clock', separate from the temperature-compensated clock generally controlling eclosion. In later papers, however, these effects were recognised as 'transient' cycles of a postulated B or 'slave' oscillator (see below).

Pittendrigh et al. (1973) demonstrated that D_2O administered in the larval medium lengthened the free-running period (see Chapter 2) but, like the effects of temperature reviewed above, only trivially so. The temperature-dependent phase angle (ψ) between the eclosion peaks and dawn, on the other hand, was more markedly affected by deuteration (Fig. 3.3). It is probable that τ is homeostatically conserved within narrow limits against all potential changes that it might encounter, this homeostasis being essential for the 'clock' function of the circadian pacemaker (see Chapter 2).

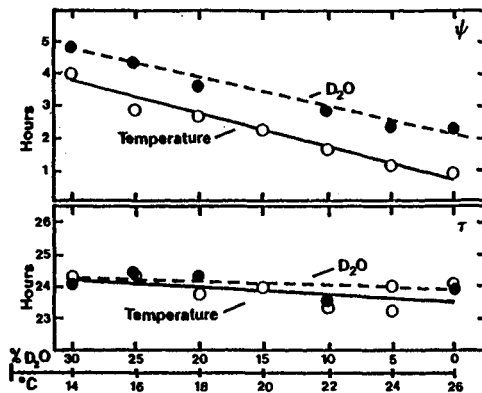


Fig. 3.3. *Drosophila pseudoobscura*, eclosion rhythm. The differential dependence of circadian period (τ) and the phase relationship of the eclosion peak to the light pulse (ψ) on temperature and D_2O . Both temperature and D_2O concentration affect τ but only slightly, suggesting that τ is homeostatically conserved in response to changes in both. For ψ , which is temperature dependent, the effects of temperature and D_2O concentration are more marked. (From Pittendrigh et al. 1973).

The arrhythmicity of eclosion of *D. pseudoobscura* born and bred in DD has been critically examined by Zimmerman (1969). It was recognised that this state might be either asynchrony between constituent oscillators (e.g. individuals in populations, cells or organs in individuals, or organelles in cells, etc.) or a true arrhythmicity in which the constituent parts were not oscillating. It was shown that a single 12-hour high-temperature pulse (20/28/20°C)

initiated a clear rhythmicity in DD populations of *D. pseudoobscura* at any time (Fig. 3.4). In a rhythmic population (transferred from LD 12:12 to DD), however, similar pulses could generate only weak phase-shifts (see Chapter 3, G. 1), insufficient to synchronise a simulated asynchronous population of flies (Fig. 3.5). The conclusion was that the aperiodic eclosion pattern seen in populations raised in DD was a case of true or primary arrhythmicity: in other words, the circadian pacemakers were inherited 'at rest' and their motion was initiated by the single temperature (or light) pulse.

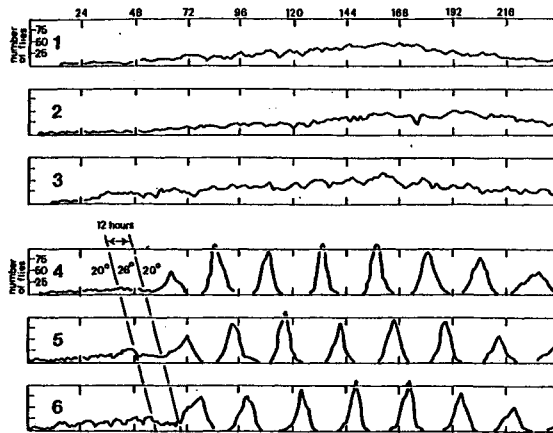


Fig. 3.4. Induction of the circadian eclosion rhythm in populations of *Drosophila pseudoobscura* by single 12-hour high temperature pulses. Populations 1, 2 and 3 were kept in constant darkness (DD) and temperature (20°C) throughout; they emerged arrhythmically. Populations 4, 5 and 6 were exposed to a single 12-hour high temperature pulse (20-28-20°C) starting at successive 8-hour intervals; subsequent eclosion was rhythmic. Time scale in hours. (From Zimmerman, 1969).

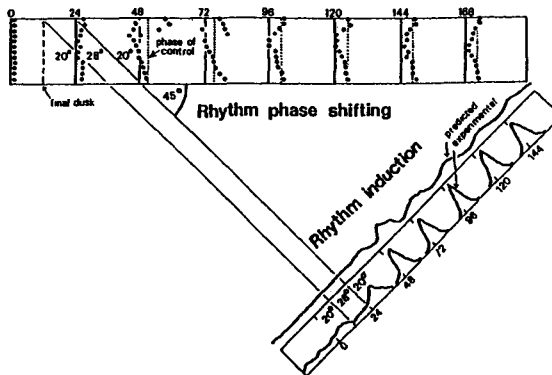


Fig. 3.5. Comparison of the rhythm-phase shifting and rhythm-inducing effects of a 12-hour high temperature pulse (20-28-20°C) on the *Drosophila pseudoobscura* eclosion rhythm. The upper panel shows the daily medians of eclosion of twelve *rhythmic* populations free-running in DD and exposed to a temperature pulse at successively later times (2-hour intervals). Forty-five degree summation of the number of flies emerging per hour (lower panel) yields the 'predicted' emergence distribution. The 'experimental' distribution, taken from FIG. 3.4 is more highly rhythmic. This suggests that the rhythmicity shown in FIG. 3.4 is brought about by an *initiation* of rhythmicity in previously arrhythmic populations rather than by phase-shifting effects on already rhythmic but out-of-phase populations. Time scale in hours. (From Zimmerman, 1969).

Figure 1 consists of two histograms. The top histogram shows the percent activity of flies over a 24-hour period, with a correlation coefficient $R=23.4$. The x-axis is labeled 'Circadian time, h' and the y-axis is labeled 'Percent'. The bottom histogram shows the activity of flies during a 16-hour gate (Gate 16) with various time intervals marked. The x-axis is labeled 'Circadian time, h' and the y-axis is labeled 'Flies'.

Pupal eclosion rhythms have also been described in a number of other insects, mainly from the Lepidoptera and Diptera; these species show many of the same general properties as *Drosophila pseudoobscura* described above. Among the Lepidoptera, eclosion rhythms have been studied in the 'giant' silk moths *Hyalophora cecropia* and *Antheraea pernyi* (Truman and Riddiford, 1970), the commercial silk moth *Bombyx mori* (Shimizu and Miura, 1987), the fall web worm *Hyphantria cunea* (Morris and Takeda, S., 1994) and the southwestern corn borer *Diatraea grandiosella* (Takeda, M., 1983). These species show the same general properties as *Drosophila pseudoobscura*. For example, the rhythm in *S. argyrostoma* free-runs in DD with τ close to 24 hours after an exposure to light-dark cycles or to continuous light (Fig. 3.6). The giant silk moths *H. cecropia* and *A. pernyi* emerge from their pupae at species-specific times of

the day. In *H. cecropia* emergence occurred in a broad peak 1 to 9 hours after dawn, whereas in *A. pernyi* it occurred later in the afternoon (Fig. 3.7) (Truman and Riddiford, 1970). As with *D. pseudoobscura*, the rhythms attained a steady-state phase relationship to the light cycle that was a function of the photoperiod. In *A. pernyi*, for example, the eclosion peak was late in the afternoon in LD 12:12, LD 17:7 and LD 20:4, but 'moved' to occupy the early hours of the night in LD 8:16 and LD 4:20. In very long photoperiods the eclosion rhythm began to break down, emergence being scattered over a wide range of clock hours; in LL it became aperiodic (Truman, 1971b). Finally, when transferred from LD 17:7 into DD, the rhythm free-ran, revealing its endogenous periodicity (τ) of 22 hours.

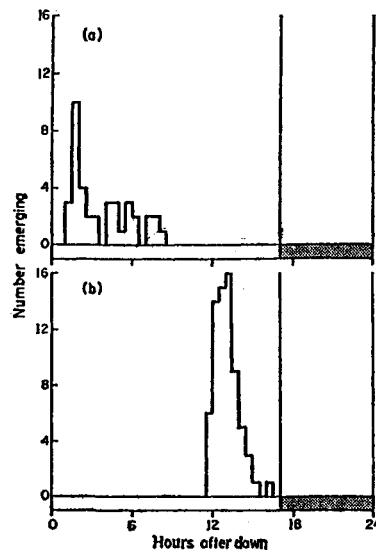


Fig. 3.7. The distribution of eclosions of the silkmoths (a) *Hyalophora cecropia*, (b) *Antheraea pernyi* in LD 17:7. (redrawn from Truman and Riddiford, 1970) (Copyright 1970 by the American Association for the Advancement of Science).

In *D. grandiosella*, *B. mori* and *H. cunea*, eclosion occurred close to dusk and the free-running period in darkness was, like that in *D. pseudoobscura*, close to 24 hours. Transfer of *B. mori* to LL led to a shortened value of τ (17.8 hours) which, however, was lengthened with a decrease in light intensity (Shimizu and Miura, 1987); in *D. grandiosella* the rhythm also persisted in LL but eclosion became arrhythmic by the third cycle (Takeda, 1983).

Pupal eclosion rhythms have been described in a large number of flies including several species of *Drosophila*: *melanogaster* (see Chapter 4), *littoralis* (Lankinen, 1986a), *subobscura* (Lankinen, 1993a) and *auraria* (Pittendrigh and Takamura, 1989; Pittendrigh et al. 1991) (see this Chapter, section I, 1). Although these species, like *D. pseudoobscura*, generally present robust eclosion rhythms, that of the related drosophilid *Chymomyza costata* has, at best, only a weak rhythmicity of low amplitude (Lankinen and Riihimaa, 1992). These authors considered that the weak eclosion rhythmicity of *C. costata* to be an adaptation for emergence at any permissive time of day around midsummer at high latitudes (69°N).

Among 'larger' flies, eclosion rhythmicity has been examined in the trypetid fruit fly *Dacus dorsalis* (Arai, 1976a), the blow flies *Calliphora stygia* (Roberts et al., 1983) and

Lucilia cuprina (Smith, 1985), the tsetse fly *Glossina morsitans* (Zdarek and Denlinger, 1995) and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1976, 1978a, 1979b). As in *D. pseudoobscura*, the rhythm in *S. argyrostoma* free-ran in DD with τ close to 24 hours after an exposure to light-dark cycles or to continuous light. In steady-state entrainment to diel light-cycles the peak of eclosion occurred close to dawn, but in LL emergence became arrhythmic. In the blow fly *Lucilia cuprina* the peak of eclosion was also close to dawn and τ_{DD} was close to 24 hours (Smith, 1985), but in the tsetse fly *Glossina morsitans* maximum eclosion centred around mid afternoon (Zdarek and Denlinger, 1995).

2. Oviposition rhythms

Oviposition rhythms have been intensively investigated in the yellow-fever mosquito *Aedes aegypti* (Haddow and Gillett, 1957), the pink bollworm moth *Pectinophora gossypiella* (Pittendrigh and Minis, 1964; Minis, 1965) and in various species of fruit-flies (Drosophilidae). More recent studies include those by Skopik and Takeda (1980) on the European corn borer, *Ostrinia nubilalis*, and Ampleford and Davey (1989) on the blood-sucking bug, *Rhodnius prolixus*. The analysis of the oviposition rhythm in *A. aegypti* by A. J. Haddow and his co-workers deserves particular attention because it was carried out - at least during its early stages - in isolation from similar studies elsewhere (Gillett, 1972).

Haddow and Gillett (1957) showed that oviposition in caged populations of *A. aegypti* occurred in well-defined peaks towards the end of the light period in a normal tropical day (LD 12:12) - both in the field and under laboratory conditions. Once again the steady-state phase of the rhythm was a function of the photoperiod: in LD 8:16, LD 12:12 and LD 16:8 the peaks occurred at the end of the light period, whereas with a shorter photoperiod (LD 4:20) the peaks moved into the dark. Populations raised in the dark showed a weak periodicity, but with as little as 5 minutes of light per 'day' a distinct rhythm became manifest (Gillett et al., 1959). Those exposed to LL, however, were completely arrhythmic (Gillett et al., 1959).

Larvae of mixed developmental ages raised in LD 12:12 and transferred to DD as adults showed a periodic oviposition pattern for at least eleven cycles (Fig. 3.8). A similar rhythm was generated by the transfer of a population from LL into DD, or by the exposure of an otherwise DD population to as little as 5 seconds of light (Gillett et al., 1961). Constant light with a single dark exposure did not elicit such a rhythm, however (Haddow et al., 1961).

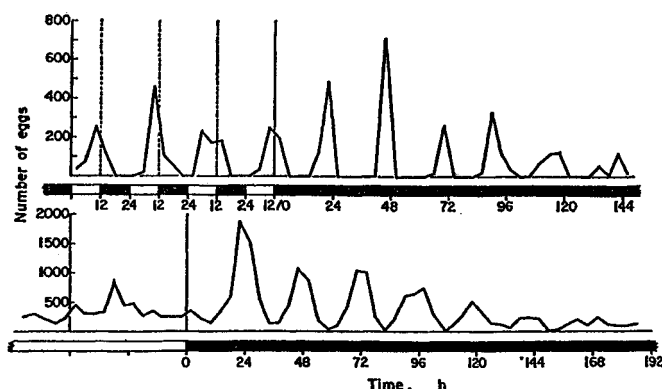


Fig. 3.8. The rhythm of oviposition in the mosquito *Aedes aegypti* showing its free-run in constant darkness (DD). (Redrawn from Haddow et al. 1961).

In the pink bollworm, *Pectinophora gossypiella*, the peak of oviposition occurred during the early part of the night and continued for about 7 hours (Pittendrigh and Minis, 1964; Minis, 1965). Unlike *D. pseudoobscura*, *A. pernyi* and *A. aegypti*, the duration of the photoperiod had little effect on the phase-relationship of the peaks to the light-cycle; Minis (1965) attributed this to the complete suppression of oviposition by the light. Transfer of the moths to DD, however, revealed an endogenous periodicity (τ) of 22 hours 40 minutes (Fig. 3.9). In the European corn borer, *Ostrinia nubilalis*, the oviposition rhythm free-ran in DD with a mean τ -value of 22.8 hours, was suppressed in LL, but like *D. pseudoobscura* was reinitiated by a transfer from LL to DD (Skopik and Takeda, 1980). In an LD cycle oviposition normally occurred at dusk and during the early part of the night.

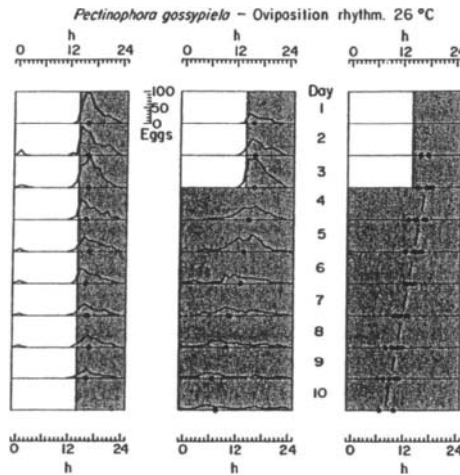


Fig. 3.9. The oviposition rhythm in the pink bollworm *Pectinophora gossypiella*. Left panel: the entrained steady state in LD 14:10, Middle panel: a free-run in DD following LD 14:10, Right panel: daily medians for seven free-runs, for which the average value of τ is 22 hours 40 minutes. (From Minis, 1965).

Considerable attention has been paid to the rhythms of oviposition in fruit-flies (Drosophilidae), including *D. melanogaster* (Rensing and Hardeland, 1967; Gruwez et al., 1971; David and Fouillet, 1973; Allemand, 1976 a, b, 1977), its close relatives (Allemand, 1974), and species of *Zaprionus* (Allemand, 1976c). The majority of these studies were confined to egg-laying behaviour in light-dark cycles (specifically LD 12:12) with little attempt to establish its persistence in constant conditions and therefore its endogeneity. In LD 12:12, Allemand (1976a, 1977) showed that the main peak of oviposition in *D. melanogaster* occurred shortly after lights-off provided the light intensity was greater than about 60 lux; below this level the LD rhythm was bimodal. Systematic dissection of female flies throughout the 24 hours, again in LD 12:12, demonstrated a cyclic variation in the development of the oocytes and a maximum retention of chorionated eggs just before the oviposition peak (Allemand, 1976b). In one of the few attempts to investigate the presumed circadian basis for this diurnal rhythmicity, Allemand (1976c) transferred flies to continuous darkness. He could find no rhythmicity in the flies reared and kept in DD, but claimed a persistent rhythm of vitellogenesis, but not oviposition, when flies were transferred from LD 12:12 to darkness.

Fleugel (1978) examined oviposition by single females of *D. melanogaster* in LD and in continuous dim light. Egg laying was found to be rhythmic in LD 12:12, but after transfer to LL the first peak of eggs could be detected but then oviposition became random. Fleugel drew attention to the fact that all other investigators had used groups of flies, and the strong social effects, coupled with the surge of oviposition following replenishment of the medium (David and Fouillet, 1973), may have accounted for much of the observed rhythmicity. In Fleugel's opinion the 'rhythm' was an hourglass (or largely exogenous in origin). Two more recent studies, however, have detected persistent rhythmicity of oviposition (albeit weak in some cases) in both wild-type and *period* mutant flies (McCabe and Birley, 1998; Sheeba et al., 2001).

In the blood-sucking bug *Rhodnius prolixus*, Ampleford and Davey (1989) showed that oviposition occurred within a narrow time close to the transition from light to darkness. The rhythm persisted in constant darkness with τ_{DD} close to 24 hours. The related species *Triatoma infestans*, *T. phyllosoma* and *Panstrongylus megistus* also showed well developed oviposition rhythms (Constantinou, 1984) with most eggs being deposited in the dark period of the cycle, close to light off in shorter nights. In *P. megistus* the rhythm free-ran in darkness with a period (τ_{DD}) of about 23 hours; in the *Triatoma* species the rhythm also free-ran through a cycle of LD 2:22 with a period longer than 24 hours.

3. Egg-hatching rhythms

Minis and Pittendrigh (1968) investigated the rhythm of egg hatching in the pink boll worm moth, *Pectinophora gossypiella*. At 20°C egg development took about 9 to 10 days, and the initial 4-hour span of oviposition (one night's egg laying) was amplified in this time to about 52 hours. Eggs raised in either LL or DD showed an aperiodic hatching pattern, but populations raised in LD 12:12 showed a distinct rhythm in which hatching was partitioned into discrete 'packets' with medians just after dawn. This rhythm could also be initiated by transfer from LD 14:10 to DD, LL to DD, or by exposure to a single non-recurrent light pulse of 15 minutes. The last two treatments consisted of signals giving no information on period; this rhythm therefore, like others reviewed, was fully innate. A single temperature pulse of 28°C, also generated rhythmicity.

One of the most interesting aspects of the egg-hatching rhythm in *P. gossypiella* was revealed by a systematic transfer of cultures from LL into DD every 5.5 hours during development. This procedure showed that the rhythm could not be initiated until after the midpoint of embryogenesis (132 hours from deposition) (Fig. 3.10). Similar data for systematic transfer from LD 12:12 to DD, or systematic exposures to 15 minutes of light, were also obtained. These results suggested that either the oscillator controlling egg hatching was not 'differentiated' until 132 hours, or that it was present from the outset but was not coupled to the light-cycle. The second of these alternatives was considered possible because a pinkish pigment appeared in the egg at about the time when initiation could first be achieved. On the other hand, the first alternative was thought to be the more plausible because a 12-hour temperature cycle also failed to initiate rhythmicity when applied to the eggs during the first half of their development. If the oscillator does not develop until this point, *P. gossypiella* differs significantly from the Queensland fruit-fly, *Dacus tryoni*, in which a transfer of circadian phase can take place through the egg from adult to larva (Bateman, 1955).

Similar egg hatching rhythms have been described in other Lepidoptera, and in some Orthoptera. For example, first instar larvae of the southwestern corn borer *Diatraea grandiosella*, and the silk moth *Antheraea pernyi*, emerged from their eggs close to dawn

(Takeda, 1983; Sauman et al., 1991); in both cases the rhythm free-ran in darkness. Eggs of the desert locust *Schistocerca gregaria* also hatched close to dawn (Padgham, 1981), but those of the cricket *Gryllus bimaculatus* hatched at night (Tomioka et al., 1991; Itoh and Sumi, 1999). In a study of six species of nemobiine crickets, Shimizu and Masaki (1997) showed that *Dianemobius fascipes* and *D. nigrofasciatus* hatched at night, whereas *Pteronemobius ohmachi*, *P. nitidus*, *D. mikado* and *D. taprobanensis* hatched within a strong peak close to dawn, with a phase angle (ψ) dependant on photoperiod.

As with *P. gossypiella* (Minis and Pittendrigh, 1968) estimations of when the system governing the egg hatch rhythm is differentiated have also been made in *G. bimaculatus* (Tomioka et al., 1991) and *A. pernyi* (Sauman et al., 1996), by systematic transfers to constant conditions as the embryos develop; in both cases this was close to mid-embryogenesis. In *A. pernyi*, period protein (PER) and timeless protein (TIM) (see Chapter 4) appeared at that time in just eight cells in the embryonic brain, suggesting the importance of these proteins in the egg hatch rhythm.

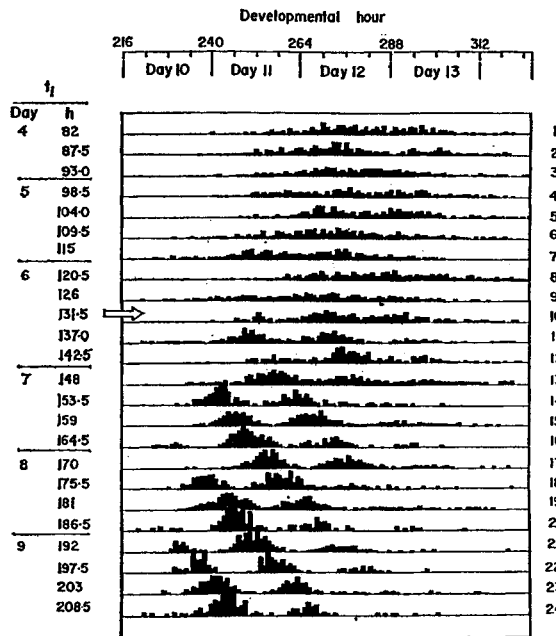


Fig. 3.10. Normalised distributions of egg hatch in continuous darkness (DD) for twenty-four populations of *Pectinophora gossypiella* eggs reared in 20°C and constant light (220 lux/m²) and systematically transferred to DD at 5½ hour intervals, from hour 82 to 208.5. The time of the transition is indicated by t_i . Note that a rhythm of egg hatch is initiated only when the LL/DD transition occurs after about the mid-point of development. (From Minis and Pittendrigh, 1968)(Copyright 1968 by the American Association for the Advancement of Science).

Although the egg-hatching rhythm of most of these species is fully innate and under circadian control, that of the tettigoniid *Metrioptera hime* (now called *Eobiana engelhardti subtropica*) is apparently not (Arai, 1977). This species shows an obligatory egg diapause, but hatches rhythmically in a daily cycle of light and temperature once 'diapause development'

(see Chapter 9) has been completed in a period of cold. However, the hatching rhythm disappeared almost immediately upon transfer to LL or DD, and also adjusted to a reversed light-cycle with none of the transient cycles observed for a circadian system. Hatching behaviour was merely triggered by lights-on or a rise in temperature: it was not coupled to the circadian system, although it may include an 'hour-glass' time-measuring process (or heavily damped oscillation?). More recent examinations of *E. engelhardti subtropica* under non-24 hour photoperiods (LD 1:1 to LD 72:72) and non-24 hour thermoperiods, have further underlined the essentially non-circadian nature of egg hatching in this species (Arai, 1998a, b). That of the related tettigoniid *Homorocoryphus jezoensis*, however, presented a clearly defined rhythm of egg hatching with peaks close to light-off, and a free-running circadian rhythm under constant conditions (Arai, 1998c).

4. Larval rhythms

Mature larvae of the Trypetidae leave fruit or their artificial breeding medium, prior to puparium-formation, with a characteristic periodicity ('larval jumping') which is under circadian control. This behaviour has been investigated in field and laboratory in a variety of species including *Dacus dorsalis* (Arai, 1975, 1976), *D. oleae* (Laudeho et al., 1978) and *Ceratitis capitata* (Myburgh, 1963; Causse, 1974). Of these, the intensive laboratory study by Causse (1974) is the most informative. Causse's study of *C. capitata* showed a remarkable similarity to the pupal eclosion rhythm of *Drosophila pseudoobscura* reviewed above.

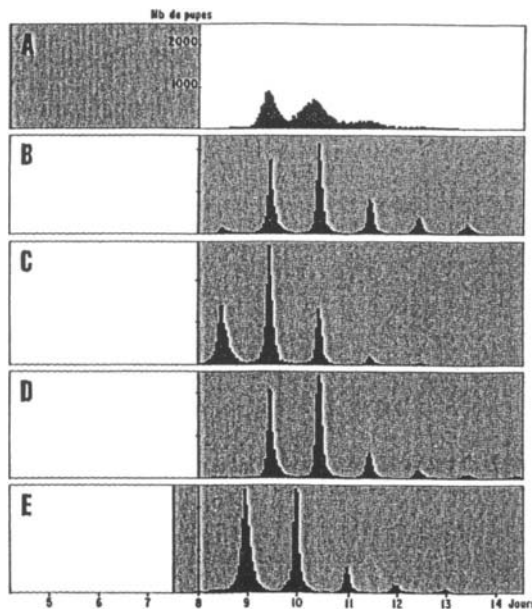


Fig. 3.11. The periodicity of larval 'jumping' (exit of mature larvae from their breeding medium) in the fruit fly, *Ceratitis capitata*, in populations transferred from DD to LL (A) or from LL to DD at various times (B to E). The rhythm free-runs in both DD and LL, but gate width is greater in LL. (From Causse, 1974).

Emergence from the larval medium occurred in a sharp peak (3 to 4 hours wide) close to dawn, but in short photoperiods (3, 6 and 9 hours per day) the peak phase-led dawn, and in long photoperiods (18, 21 and 23 hours per day) it phase-lagged it. There was also an exogenous response at lights-on. The rhythm free-ran in DD with a near 24-hour periodicity after LD or LL; it also persisted in LL but with an increased peak width and a tendency towards arrhythmicity (Fig. 3.11). Single step-wise transfers from LL to DD generated a clear rhythmicity at all stages of larval development (Fig. 3.12). Furthermore, a single light-pulse induced rhythmicity in otherwise poorly rhythmic DD cultures, although the 'amplitude' of the peaks was lower when the initiating pulse of light was less than 6 hours. Causse also demonstrated a rhythm in the sex ratio (female per cent) among the flies emerging from their puparia.

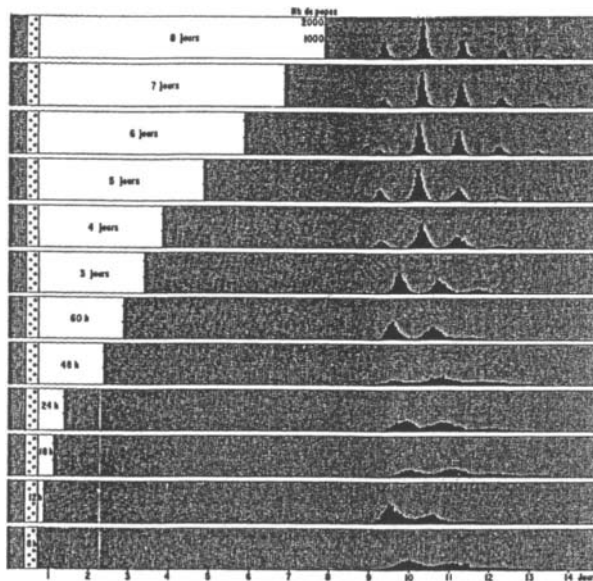


Fig. 3.12. *Ceratitidis capitata*. The circadian rhythm of larval 'jumping' in cultures transferred from LL to DD at different stages of development. The dotted areas on the left represent an 8-hour oviposition period for the populations concerned. Rhythmicity is generated in all cultures, but gate width is narrower with those transferred to DD later in larval life. (From Causse, 1974).

Rhythms of mature larvae leaving their larval site (larval 'wandering' behaviour) have also been described in blow flies, flesh flies, tsetse flies, and in the fruit fly *Drosophila melanogaster*. Larval wandering in the blow fly *Lucilia cuprina* (Smith et al., 1981) and the flesh fly *Sarcophaga argyrostoma* (Richard et al., 1986) was found to occur in a nocturnal peak but with only a weak persistence in DD. However, in a later study with *L. cuprina*, Warman (1995) demonstrated a more robust free-running rhythm with a period (τ) of 23.4 hours. With the fruit fly *Drosophila melanogaster*, Roberts et al. (1987) showed wandering from the food during the night, but no discernible persistence in LL or in overcrowded cultures. The endogeneity of this rhythm in *D. melanogaster*, therefore, remains to be established.

In the viviparous tsetse fly *Glossina morsitans* larviposition or parturition was found to occur late in the afternoon (Denlinger, 1983; Robinson et al., 1985; Zdarek et al., 1992). Transfer of the population to constant light led to a rhythm that persisted for about two to three cycles. Denlinger (1983) investigated the contributions made by mother and intra-uterine larva to this rhythm by placing pregnant females in 'divided chambers' in which the mother's head was in one condition (LD 12:12 or LL) and the tip of the abdomen, and hence the larva, was in the opposite condition. Denlinger concluded that both mother and intra-uterine larva contributed to the timing of parturition.

Larval moults in various species may occur at particular times of the day or night. In the silkworm *Antheraea pernyi* and the tobacco hornworm *Manduca sexta*, for example, ecdyses occur in sharp peaks. Truman (1972a), however, demonstrated that the moults themselves were not controlled directly by the circadian system; they occurred at certain time intervals after releases of brain hormone, which were gated events (see Chapter 8, B). The time interval between hormone release and ecdysis was instar-specific and temperature dependent. In a subsequent flurry of papers using similar techniques, endogenous gated rhythms of hormone release have been described for larval-larval and larval-pupal moults in the silkworm *Samia cynthia ricini* (Fujishita and Ishizaki, 1981, 1982a, b) and the commercial silk moth *Bombyx mori* (Sakurai, 1983, 1984). These rhythms will be considered further in Chapter 8.

5. Pupation rhythms

Circadian rhythms of pupation are apparently frequent in mosquitoes (Nayar, 1968; McClelland and Green, 1970; Reiter and Jones, 1975). Pupation in the salt-marsh mosquito *Aedes taeniorhynchus*, for example, has been intensively studied by Nayar (1967 a, b; 1968), Provost and Lum (1967) and Lum et al. (1968).

In *A. taeniorhynchus* the daily peaks of adult emergence are *not* clock-controlled but merely reflect the antecedent rhythm of pupation; the interval between pupation and eclosion, for example, is dependent on temperature (Nielsen and Haeger, 1954; Provost and Lum, 1967). Nayar (1967 a, b) showed that larval cultures raised from the egg stage in DD showed a faint sinusoidal rhythm of pupation with a period (τ) of about 21.5 hours at both 27°C and 32°C. When a single light pulse of 4 hours was applied at the beginning of the last larval instar the peaks became more pronounced - as though the pulse had served to synchronise previously random oscillators - and τ became 22.5 hours. Since a single light-pulse contains no information on period, and the frequency was unaffected by temperature, the rhythm of pupation was considered to be endogenous and temperature-compensated. A similar effect was observed following a single temperature pulse of 4 hours at 32°C in an otherwise 27°C culture. The most curious feature of this rhythm was that it could not be entrained to a 24-hour cycle of light (LD 12:12) or temperature (12 hours at 27°C, 12 hours at 32°C) and continued to run with a periodicity of about 22.5 hours (Provost and Lum, 1967; Nayar, 1967a). Only when the larvae were reared in the 'stress' conditions of crowding, high salinity and 'basic' ration was the period of the rhythm close to 24 hours (Nayar, 1967b). One explanation for this inability to lock onto the exact 24-hour periodicity of the solar day could be that the clock became 'uncoupled' from the light-cycle before the pupal ecdysis so that the pupation rhythm was actually free-running in LD 12:12 as though it were in DD. Nevertheless, the larvae were clearly sensitive to light signals since reversed light-cycles or single light perturbations caused phase-shifts with observable transients, continuous light (LL) led to arrhythmicity within 48 hours, and the rhythm could apparently be entrained to environmental light cycles shorter than 24 hours. In LD 11:11, LD 8:8 and LD 6:6, for example, the rhythm adopted a period of 22,

20, 16 and 12 hours, respectively (Nayar, 1968). How far this can be considered normal entrainment, as opposed to an exogenous effect, is not clear.

The mosquito *Anopheles gambiae* also possesses a well-defined rhythm of pupation (Jones and Reiter, 1975) which, like that in *Aedes taeniorhynchus*, confers rhythmicity on subsequent adult eclosion. Reiter and Jones (1976), however, have shown that 'fine adjustment' of eclosion timing is possible in this species through light-mediated entraining effects on the eclosion system itself.

Outside the mosquitoes, clock-controlled pupation (and pupariation) seems to be rather uncommon. An outstanding example is puparium formation in *Drosophila victoria* (Rensing and Hardeland, 1967; Pittendrigh and Skopik, 1970). In contrast, puparium formation in the flesh fly *Sarcophaga argyrostoma* is not a gated event (Richard et al., 1986); it occurs at any time during the day or the night, even though larval wandering and subsequent pupal eclosion are under clock control (Saunders, 1986). These differences must surely depend on the selective advantages of wandering and eclosion 'at the right time of the day', whereas puparium formation occurs only when the larvae have become subterranean and timing becomes unimportant.

Among Lepidoptera, pupation in the southwestern corn borer *Diatraea grandiosella* occurred in a peak at dusk which then free-ran in both DD and LL (Takeda, 1983). The pupation peak, however, was less synchronised than either egg hatch or pupal eclosion, and in LL pupation became arrhythmic by the third cycle. Pupation itself was not a gated event in the silk moths *Samia cynthia ricini* and *Bombyx mori* although the endocrine events leading up to the last larval-pupal moult were dictated by a circadian clock (Fujishita and Ishizaki, 1982; Sakurai, 1984) (see Chapters 5 and 8).

B. POPULATION RHYTHMS AS 'GATING' PHENOMENA

The general properties of the pupal eclosion rhythm in *D. pseudoobscura* suggest the existence of a self-sustained oscillator that partitions a mixed-age population into daily activity peaks. This further suggests that certain hours of the day constitute 'forbidden zones' for eclosion, and that the 'allowed zones' or peaks are dictated by the circadian clock. Pittendrigh (1966) has called these allowed zones 'gates'.

The validity of using a mixed-age population of insects to demonstrate an endogenous rhythm has been questioned, however. Harker (1964, 1965 a, b) related the timing of certain developmental events (head eversion, eye and wing pigmentation) in the pupa and pharate adult of *D. melanogaster* to the time of adult emergence on the one hand and to the environmental light-cycle on the other. According to Harker the timing of these developmental events varied widely according to the circadian time at which the individual commenced an earlier stage in its development. For example, the interval between head eversion and the appearance of the yellow eye pigment was shorter when the earlier event occurred during the hours shortly after dawn than at later times in the cycle. She concluded that a fly simply emerged when its development was completed, and that its developmental time was only the sum of all the intermediate developmental stages. She further concluded that the observed rhythm of eclosion was not a function of an oscillation in an individual. However, her results would suggest - even if eclosion were not controlled by a circadian clock - that the intermediate 'steps' were.

Work by Skopik and Pittendrigh (1967) and Pittendrigh and Skopik (1970) with both *D. pseudoobscura* and *melanogaster*, however, failed to substantiate Harker's findings, and these authors arrived at quite a different conclusion. Using developmentally synchronous

populations of flies collected as newly formed puparia in a 1-hour 'collection window' (at t_0), Pittendrigh and Skopik (1970) examined the times of the following events within the puparium: head eversion (t_h), yellow eye pigmentation (t_y), black pigmentation in the ocellar bristles (t_b), and the final act of eclosion (t_e). In the absence of a circadian oscillation (i.e. in continuous high-intensity light) the distribution of each developmental stage occurred as a single peak (Fig. 3.13), although the initial range of 1 hour during which puparium-formation had occurred had widened to 20 hours or more by the time of emergence. The rate of development was, of course, temperature-dependent, and the females completed their development before the males.

In the presence of a circadian oscillation, generated either by a transfer from LL to DD, or by an alternating cycle of LD 18:6, the picture was quite different. Figure 3.14 shows the fate of twenty-seven initially synchronous populations which experienced the LL/DD transition at different developmental ages. The appearance of yellow eye pigmentation and black ocellar bristles occurred at fixed time intervals after puparium formation. The time of eclosion, however, depended on the circadian time at which the transfer to DD occurred, and in some populations the distribution was clearly 'split' into two distinct peaks, almost 24 hours apart.

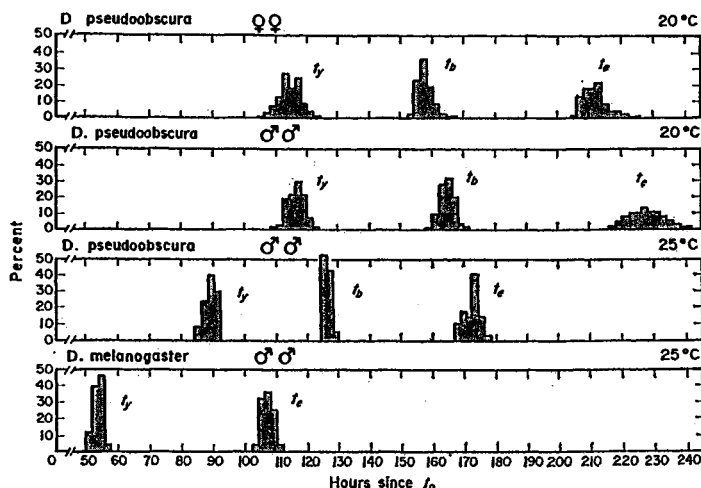


Fig. 3.13. The time course of emergence and of some prior developmental events in populations of *Drosophila pseudoobscura* which formed puparia within a known 1-hour interval ($t_0 \pm 0.5$ hours) and maintained throughout at 20 or 25°C and continuous bright white light (LL). t_y = time of appearance of yellow eye pigmentation; t_b = black pigment appearing in ocellar bristles; t_e = time of eclosion. Note that the distribution of these events becomes wider, but remains unimodal. (From Pittendrigh and Skopik, 1970).

In LD 18:6 these peaks occurred, as might be expected, in the hours that immediately follow 'dawn'. The results showed that if the developing adults were not at the 'correct' morphogenetic stage to utilise one particular allowed zone or gate, they were required to remain within the puparium until the next, the intervening hours constituting a 'forbidden zone' for eclosion. In *D. pseudoobscura* the gates recurred with circadian frequency (modulo $\tau + 15$ hours) after the LL/DD transition. A more recent study using *D. melanogaster* (Qui and Hardin, 1996) has described a similar interaction between the rate of development and the use of particular eclosion gates.

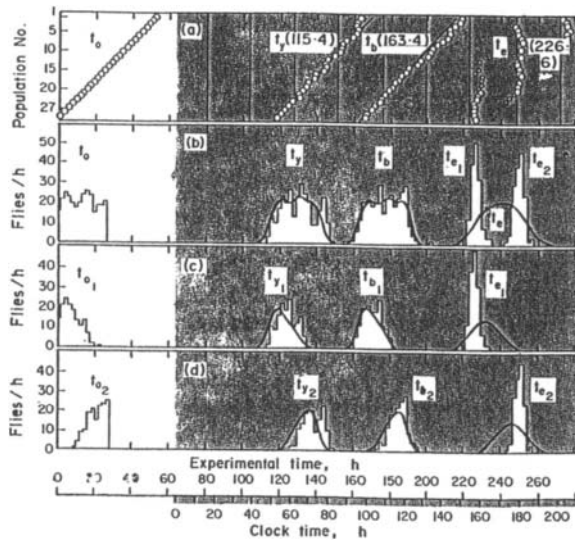


Fig. 3.14. Gated and ungated events in the development and emergence of *D. pseudoobscura* (adult males) after a single step from LL to DD at different developmental times. The top panel (a) plots the medians for twenty-seven individual populations of developmentally synchronous pupae. Note that in certain populations the distribution of eclosions is partitioned into two discrete peaks about 24 hours apart, whereas those for the intermediate developmental steps are not. (From Pittendrigh and Skopik, 1970)(see Fig. 3.13 for symbols).

These results demonstrated the reality of an endogenous oscillation in each developing fly that dictates the circadian time of eclosion but not that of the intervening developmental stages. In an earlier paper (Skopik and Pittendrigh, 1967; see also Pittendrigh, 1966) it was clearly demonstrated how a series of developmentally synchronous populations, in which the LL/DD transition was systematically varied, could be rearranged to simulate a population of mixed developmental age (Fig. 3.15).

The gating of certain developmental stages by such a mechanism is probably ubiquitous in insect 'population' rhythms. It is evident, for example, in the pupal eclosion rhythm of *Antheraea pernyi* (Truman, 1971a), egg hatch (Minis and Pittendrigh, 1968) and oviposition of *Pectinophora gossypiella* (Pittendrigh and Minis, 1964), and in the oviposition rhythm of *Oncopeltus fasciatus* (Rankin et al., 1972). (See also the gating of the circannual rhythm in the beetle *Anthrenus verbasci* [Blake, 1958, 1959; Chapter 15]). McClelland and Green (1970) described a situation in the mosquito *Aedes vittatus*, in which the rhythm of pupation was so controlled. In these experiments virtually synchronous larval cultures were obtained by immersing eggs on filter paper into water for a 3-hour period. Eight such cultures were established, the start of each being staggered over a 24-hour period in 3-hour intervals. Figure 3.16 shows that larvae reared in LL showed no periodicity in pupation, as expected, males developing in about 67 to 69 hours and the females in 72 to 76 hours. Under a regime of LD 12:12, however, the larvae were clearly forced to utilise pupation 'gates' only available at certain phases of an on-going oscillation. Under certain circumstances, the populations were partitioned into two discrete pupation peaks. In *A. vittatus* the width of each gate was about 17 hours for males and about 15 hours for females; this contrasts with a gate width of only 6 hours for both sexes in *D. pseudoobscura* (Skopik and Pittendrigh, 1967). It is of interest, however,

that the females with their longer developmental time were sometimes forced to utilise a later gate than the males. In *D. pseudoobscura* the converse was true because the males showed a longer development. A similar periodicity of pupation was described for *Aedes taeniorhynchus* (Neilsen and Haeger, 1954), but was not apparent in *A. aegypti* (McClelland and Green, 1970).

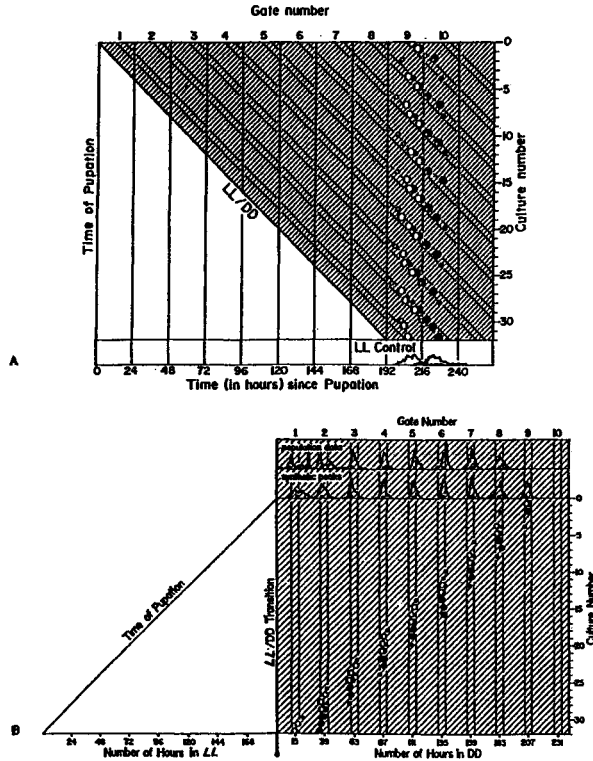


Fig. 3.15. A – the effect of an LL/DD transition on the time of emergence of adults of *D. pseudoobscura* from populations of the same developmental age. The medians of the emergence peaks are given as points; open (female) and solid (males). For some cultures (e.g. No. 1, female) there is only one emergence peak. For others, there are two (e.g. No. 2, female); the median points for these cultures are shown half size. B – simulations of a population of mixed developmental ages, by rearrangement of the data from A. The uppermost panel (labelled 'population data') gives the observed distribution of emergence peaks and their medians for a population of pupae of mixed developmental age that had been cultured in LL and transferred to DD at the point indicated. The next panel ('synthetic peaks') was obtained by summing, and normalising to equal areas, all the distributions whose medians are given below as open and solid points. Thus the 'synthetic peaks', like those marked 'population data'. Include both sexes. (From Pittendrigh, 1966).

Although pupation in *A. aegypti* is not a periodic or circadian phenomenon, egg deposition is so controlled (Haddow and Gillett, 1957). Gillett (1962) studied the 'contribution' made by individual females to the population rhythm by enclosing them in small lamp-glass cages and observing the times of feeding and oviposition for each insect. In a natural tropical light-cycle of LD 12:12 Gillett showed that each mosquito that had completed egg development 'waited' for the first available egg-laying period. If all her eggs were mature when the first gate arrived all were deposited in a single batch. On the other hand, some

females were forced to deposit some of their eggs in one gate and the remainder in the next, a full 24 hours later. A similar situation was observed for mosquitoes transferred from LL to DD, but in LL eggs were deposited when mature and with no sign of periodicity. When transferred to DD the first gate occurred 22 hours after the LL/DD transition and at circadian intervals thereafter. Following the terminology adopted for *D. pseudoobscura* (Pittendrigh, 1966), therefore, the gates were observed to occur at intervals of modulo $\tau + 22$ hours, where τ for *A. aegypti* was close to 24 hours. Since the rate of egg development was temperature-dependent whereas the period of the oscillation controlling the rhythm was not, mosquitoes kept at a lower temperature were forced to utilise later gates (Gillett, 1972).

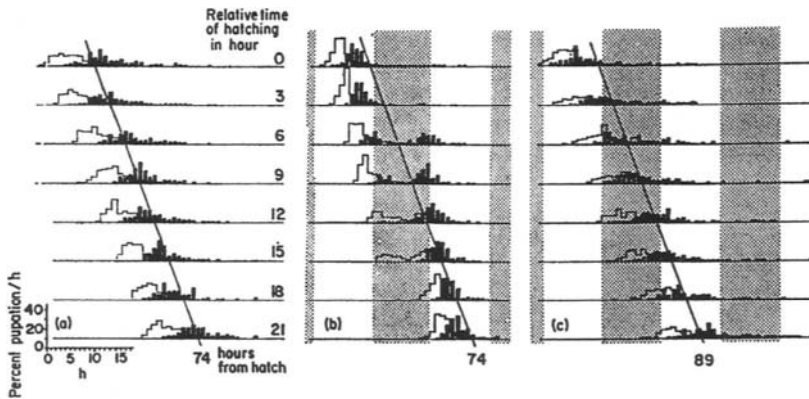


Fig. 3.16. The distribution of pupation times in sequentially hatched populations of mosquito larvae. Each successively lower line represents a population hatched three hours later than the one above and reared at 31°C under the light regime indicated by shading. The hollow histograms represent the percentage of males pupating per hour; the solid bars represent females. The oblique line cuts each base line at the same age for each population and represents the mean time of pupating of females in the eight populations pooled. (a) *Aedes vittatus* in LL; (b) *A. vittatus* in LD 12:12; (c) *A. aegypti* in LD 12:12. Note how the distribution of pupation of *A. vittatus* in LD 12:12 is partitioned in some populations into discrete peaks or 'gates', whereas pupation in *A. aegypti* is not a gated event. (From McClelland and Green, 1970).

C. ENTRAINMENT OF POPULATION RHYTHMS BY LIGHT

The entrainment of a free-running circadian oscillator by the solar day *Zeitgeber* of light (and temperature) involves daily corrections of τ to T and the attainment of a particular phase relationship (ψ) between the overt event (e.g. the eclosion maximum) and the light-cycle. It is the latter, in particular, which is of biological significance to the organism: it enables the circadian clock, for example, to time events to the 'right' part of the day.

Pittendrigh (1974, 1981a) distinguished between two broad classes of entrainment, parametric and non-parametric. Parametric entrainment involves a continuous modulation of the circadian pacemaker's 'angular velocity' by the *Zeitgeber*, throughout all or only part of its cycle (i.e. the light pulses speed up or slow down the motion of the pacemaker at different phases). Non-parametric entrainment, on the other hand, involves almost instantaneous phase shifts to correct τ to T , frequently in response to quite short light-pulses. It seems almost certain that the two types represent ends of a continuous 'spectrum', and that particular cases may contain elements of both. Entrainment of circadian oscillators by fluctuating cycles of

light intensity in the sub-arctic summer, for example, or entrainment by long light-pulses, must involve parametric entrainment, whereas the abrupt and effectively discrete phase-shifts described for *Drosophila pseudoobscura* in this chapter must be largely non-parametric. The difference probably reflects the speed of the changes in phase, which in turn may reflect the sensitivity of the photoreceptor and/or phase-shifting mechanism, and the 'strength' (duration and intensity) of the resetting light pulses.

In this section we will examine the non-parametric model for entrainment which has been developed for *D. pseudoobscura* (Pittendrigh, 1965, 1966). This model is based on the phase-shifting properties of single pulses of light perturbing the free-running oscillation in DD; the phase-response curves so produced may be used to calculate and predict all formal aspects of the phenomenon with considerable precision.

1. The phase-response curve

When the eclosion rhythm of *D. pseudoobscura* is allowed to free-run in DD after exposure to LD 12:12 or to LL it is regarded as passing through alternate half-cycles of 'subjective night' and 'subjective day', with the beginning of subjective night (called hereafter circadian time, Ct 12) occurring at the LL/DD transition and at intervals of (modulo τ) thereafter (Pittendrigh, 1965). Since τ for *D. pseudoobscura* is essentially 24 hours, subjective night and day each occupy $\tau/2$ or 12 hours. In such a free-running state the peaks of eclosion occur at interval of modulo $\tau + 15$ hours after the LL/DD transfer. However, single pulses of white light applied at different phase points of the oscillation, i.e. at different circadian times, will produce substantial phase-shifts in the subsequent steady state of the rhythm. For example, 15-minute light pulses applied early in the subjective night cause significant phase-delays ($-\Delta\phi$), whereas those applied late in the subjective night or early in the subjective day produce significant phase-advances ($+\Delta\phi$). Pulses applied between Ct 4 and Ct 12, however, have little or no effect on the phase of the resulting rhythm of eclosion, giving the so-called 'dead zone', and at Ct 18 there is an abrupt 360° change from phase-delay to phase-advance. These responses to single light perturbations provide a phase-response curve for the oscillation (Fig. 3.17).

Although the concrete nature of the oscillation in *D. pseudoobscura* remains incompletely known (see Chapter 4), as it is in all other organisms, the phase-response curve provides one experimental assay of the oscillation's phase, and is the best available characterisation of its time-course.

The 'standard' phase response curve shown in Fig. 3.17 illustrates the responses of *D. pseudoobscura* to single 15-minute pulses of white light of about 500 lux. For each species, however, there is a 'family' of PRCs depending on the strength (i.e. duration and intensity) of the perturbing light-pulse. An early demonstration of this fact involved the determination of phase response curves for *D. pseudoobscura* exposed to pulses of 12 hours, 4 hours and 1/2000 seconds (Pittendrigh, 1960); these curves differed mainly in the magnitude of the $\Delta\phi$ produced, and in the circadian time of the phase inversion.

Also working with *D. pseudoobscura*, Engelmann (1969) examined the phase-shifts evoked by blue light pulses (the most effective region of the spectrum; see Chapter 3, H) between 1/100 second to 15 minutes duration, and of 0.5 to 5000 ergs cm⁻², at two circadian phases: Ct 16 where phase delays are produced and Ct 20 for phase advances. It was found that the magnitude of $\Delta\phi$ was a function of signal energy: no significant effects were noted up to 10² ergs cm⁻², but full phase-shifts were achieved with energies above 10⁴ ergs cm⁻².

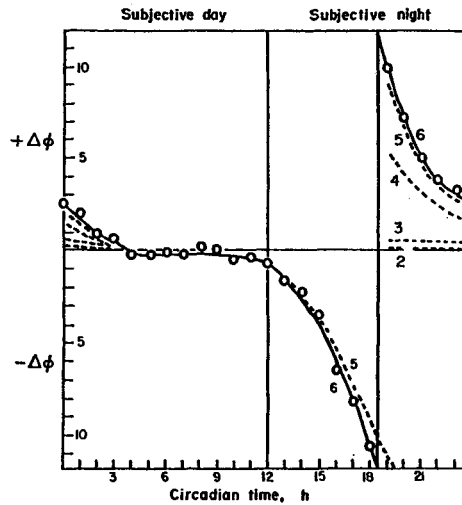


Fig. 3.17. The phase response curve for the *Drosophila pseudoobscura* eclosion rhythm based on 15 minute signals of white light. The solid curve and the points plotted as open circles, describe the steady state phase shifts measured on day 6 after the signal. The curves plotted as dotted lines are based on the observed phase shifts on days 2, 3, 4 and 5. Delays ($-\Delta\phi$) are essentially completed immediately, but advances ($+\Delta\phi$) develop slowly and are not complete until day 5 or 6. (Redrawn from Pittendrigh, 1965).

In a series of papers, Winfree (1970b, 1971, 1972a) systematically examined the responses of the *D. pseudoobscura* eclosion clock to pulses of dim blue light ($10 \mu\text{W cm}^{-2}$) of varying duration. Populations of developing flies were transferred from LL to DD to generate a rhythm, and then tested at intervals with the light pulse. He found that when pulse lengths were less than about 50 seconds the phase shifts effected were relatively small ($<4\text{-}5$ hours); the resultant steady-state phase response curve was only slightly different from the unperturbed control, and crossed the control line twice per cycle. Pulses greater than 50 seconds, on the other hand, gave rise to large phase shifts (up to 12 hours), and the resulting response curve crossed the control line only once (Fig. 3.18). When intervals (θ) between the resetting pulses and the eclosion peaks were plotted against time (T) elapsed since the LL/DD transition, short pulses gave rise to a resetting curve with an average slope of 1, whereas large pulses gave an average slope of 0 (Fig. 3.18). Winfree therefore called these types 'weak' or Type 1, and 'strong' or Type 0. A fuller account of this work and its significance will be given later (Chapter 3, E).

The two types of PRC have been observed in several other insect species. In *D. melanogaster*, for example, 15-minute light pulses gave a weak Type 1 curve with advances and delays never exceeding 3 hours (Ottesen et al., unpublished), and with the *Sarcophaga argyrostoma* eclosion rhythm, pulses of white light ($240 \mu\text{W cm}^{-2}$) of less than about 4 hours gave rise to low 'amplitude' Type 1 curves, whereas pulses longer than 4 hours gave rise to strong Type 0 curves (Saunders, 1976, 1978a) (Fig. 3.19). Working with the oviposition rhythm in the European corn borer *Ostrinia nubilalis*, Skopik and Takeda (1980) found that 1 hour pulses of light gave a Type 1 PRC, whereas 4 hour pulses gave Type 0. And in the eclosion rhythm of the silk moth *Bombyx mori*, pulses longer than 2 hours gave Type 0 (Shimizu and Miura, 1987). Eclosion rhythmicity in the blow fly *Lucilia cuprina* (Smith, 1985) also showed a change from Type 1 to Type 0 as the perturbing light pulses ($100 \mu\text{W cm}^{-2}$) increased in duration from 1 to 8 hours.

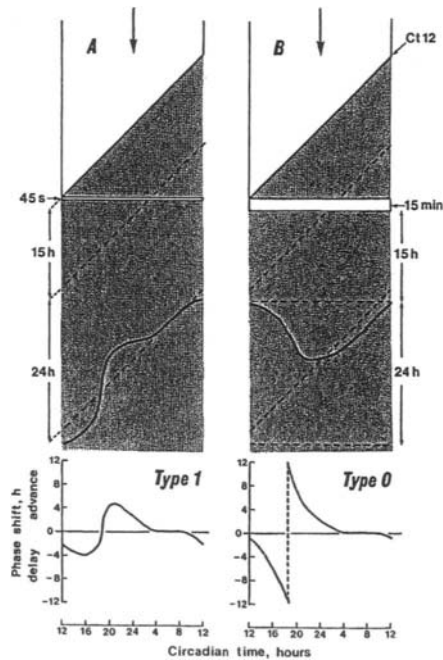


Fig. 3.18. Winfree's (1970) Type 1 and Type 0 resetting curves for a circadian rhythm (data taken from the eclosion rhythm of *Drosophila pseudoobscura*, various sources). A – 'weak' (= short) light pulses (45 seconds); B – 'strong' (= long) light pulses (15 minutes). In the upper panels the time courses run vertically downwards. Different cultures of flies are transferred from LL to DD (phase equivalent to Ct 12) and then experience a single light pulse (either 45 seconds or 15 minutes) at different circadian times. The heavy line plots the steady state phase relationships of the eclosion peaks. Dotted diagonal lines indicate times occurring $15 + \text{modulo } \tau$ hours after transfer from LL to DD; horizontal dotted lines indicate times occurring $15 + \text{modulo } \tau$ hours after the end of the resetting pulse. In Type 1 responses (A) the reset curve has an average slope of 1 and is little disturbed from the unperturbed control (diagonal dotted line). In Type 0 responses (B) the reset curve has an average slope of 0. (Experimental format after Winfree, 1970). In the lower panels Type 1 and Type 0 responses are shown as conventional phase response curves (Format after Pittendrigh).

Type 0 curves are apparently widespread in 'population' or 'developmental' rhythms, provided energy levels are high enough; and, of course, where Type 0 curves are found, Type 1 responses to weaker signals must occur. The reverse situation, however, does not necessarily follow. For example, Type 1 PRCs which are characteristic for 'behavioural' rhythms such as cockroach (Chapter 2) and rodent activity (Pittendrigh and Daan, 1976) do not always give way to high 'amplitude' Type 0 curves when the energy of the perturbing pulses is increased (see, however, the cockroach case, Chapter 2, D.2).

The 'standard' Type 0 PRC for *D. pseudoobscura* as represented in Fig. 3.17 is based on steady-state phase shifts ($\Delta\phi$) of the rhythm which, especially in the case of phase advances, are only reached after several so-called transient (i.e. non-steady-state) cycles. Although some theoretical models for the circadian clock show that a single oscillator can undergo such transients after perturbation (see, for example, Klotter, 1960; Johnsson and Karlsson, 1972a), transients may also be accounted for by a two-oscillator model proposed by Pittendrigh and Bruce (1957, 1959) and Pittendrigh et al. (1958).

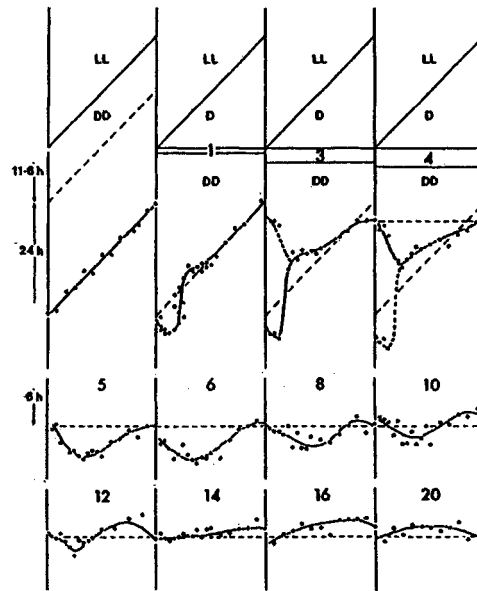


Fig. 3.19. Resetting curves for the pupal eclosion rhythm in the flesh-fly *Sarcophaga argyrostoma* exposed to single pulses of white light ($240 \mu\text{W cm}^{-2}$). All cultures run vertically out of LL into DD (as in Fig. 3.18) and then experience a single resetting pulse of 1 to 20 hours duration. Plotted points show eclosion medians occurring either $11.6 + \text{modulo } \tau$ hours after the LL/DD transition, or $11.6 + \text{modulo } \tau$ hours after the end of the resetting pulse. Pulses of 1 and 3 hours are considered to give rise to 'weak' Type 1 responses; those of 5 hours or more to 'strong' Type 0 responses. Pulses of 4 hours duration give ambiguous results. (Original).

The essential features of this model are that the physiological mechanism immediately underlying eclosion is governed by one oscillator (the B-oscillator) that is distinct from a second and light-sensitive A-oscillator. The A-oscillator is envisaged as a central *pacemaker* directly coupled to the environmental light-cycle. The B-oscillator, on the other hand, is regarded as a driven (or *slave*) element, coupled to the driver (A) and whose phase more immediately controls eclosion. It is further envisaged that the A-oscillator is immediately reset by the light pulse but the B-oscillator requires several cycles before it attains a steady-state relationship to the driver; hence the series of transients. The B-oscillator is not light sensitive, but may be sensitive to temperature pulses and cycles. B therefore derives phase-control from two sources - directly from the temperature cycle and indirectly, via A, from the light-cycle.

For several reasons it is convenient to represent the phase-response curve in a mathematically equivalent form in which the advances are displaced 360° (24 hours of circadian time) on the ordinate and treated as phase delays, thus yielding a monotonic curve (Pittendrigh, 1967) (Fig. 3.20). Figure 3.21 shows three cycles of the oscillation following an LL/DD transition in this monotonic form, and illustrates the terminology adopted by Pittendrigh (1965, 1966, 1967b) to describe the parameters of the system.

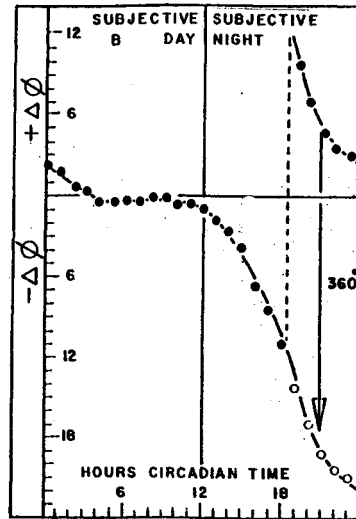


Fig. 3.20. The phase response curve for the eclosion rhythm of *Drosophila pseudoobscura* showing its monotonic form. (From Pittendrigh, 1967).

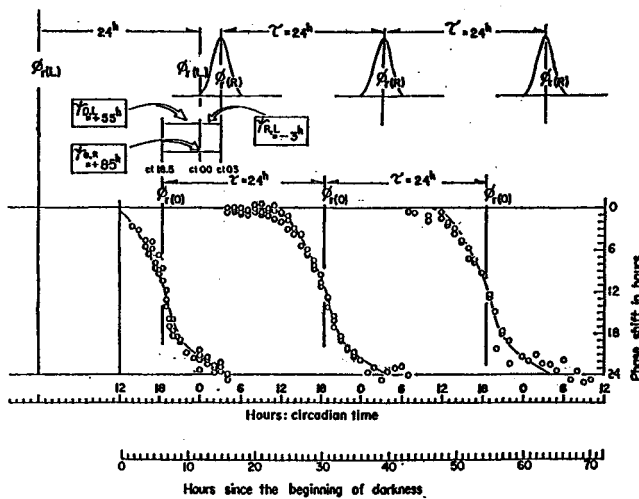


Fig. 3.21. The phase response curve for the *D. pseudoobscura* eclosion rhythm, in its monotonic form, for three full cycles (72 hours) of the oscillation's free run in DD following LD 12:12. The oscillation begins at Ct 12 when the lights go out. Each point plots the phase shift as a delay ($-\Delta\phi$) caused by a single 15 minute light pulse at the time indicated on the abscissa. ϕ_r = phase reference point; $\phi_r(L)$ = phase reference point of the light cycle; $\phi_r(R)$ = phase reference point of the rhythm (median of eclosion peak); $\phi_r(O)$ = phase reference point of the oscillation; $\psi_{R,L}$ = phase angle (time) difference between the reference points of rhythm and light cycle; $\psi_{R,O}$ = phase angle difference between the reference points for rhythm and oscillation; $\psi_{O,L}$ = phase angle difference between reference points for oscillation and light. (From Pittendrigh, 1967).

As a test of Pittendrigh and Bruce's hypothesis that light causes virtually instantaneous resetting of the A-oscillator, Chandrashekaran (1967a) transferred cultures of *D. pseudoobscura* from LD 12:12 to DD and then exposed them to two pulses of light (15 minutes at 3000 lux) in the same circadian cycle. In one experiment, the first pulse (P_1) was timed to occur 27.5 hours after entry into DD and the second (P_2) at 34.0 hours after DD. According to the phase-response curve for 15-minute perturbations (Fig. 3.17), the first pulse would fall at Ct 15.5 and cause a phase-delay ($-\Delta\phi$) of 5.0 hours in steady state; the second pulse, in isolation, would fall at Ct 22.0 and cause a phase advance ($+\Delta\phi$) of 4.8 hours. The result, however, indicated that virtually instantaneous resetting had indeed occurred after P_1 . The 5-hour $-\Delta\phi$ caused by P_1 resulted in P_2 falling, not at Ct 22.0, but at Ct 17.0 where it caused a further $-\Delta\phi$ of 8 hours; the net observed phase-delay of 13.5 hours was very close to the theoretically expected delay of 13.0 hours. In a second experiment, the opposing influences of pulses occurring 27.5 hours and 38.5 hours after DD cancelled themselves out because after the instantaneous $\Delta\phi$ caused by P_1 ($-\Delta\phi = 5.0$ hours) P_2 fell at Ct 21.5 and caused an advance ($+\Delta\phi$) of roughly the same magnitude.

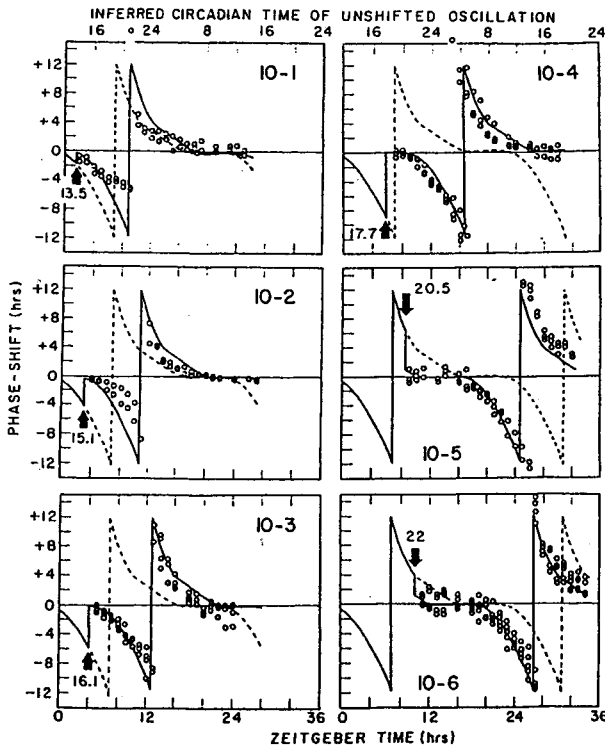


Fig. 3.22. *Drosophila pseudoobscura*, eclosion rhythm. Assay of the pacemaker's time course following a phase shift ($\Delta\phi$) in the first circadian cycle following a transfer from LD 12:12 to DD. The action of the first pulse (heavy arrow) is assayed by the $\Delta\phi$ response to a second 15-minute pulse (see text for details). Solid curve (PRC) indicates predicted time course of the pacemaker before and after exposure to pulse 1. The dotted curve indicates the course it would have followed if unperturbed. Time courses are close to prediction, except for first pulses beginning in the first 4 hours of darkness. (From Pittendrigh).

Pittendrigh (1974, 1981a) subsequently provided further strong documentation for the essentially instantaneous resetting caused by single 15-minute light pulses, again by using two such pulses per circadian cycle. In these experiments, populations of flies were transferred from LL to DD to initiate rhythmicity. Twelve separate sets (of 25 populations each) then received a first pulse at different times after the LL/DD transition, and each of the 25 sub-groups a *second* pulse at intervals thereafter. The second pulses were thus used to establish phase-response curves to determine whether the first pulse had evoked the expected shifts. Figure 3.22 shows that in all but a few notable exceptions the results were in full agreement with prediction: the first pulses had induced virtually instantaneous phase shifts as in the non-parametric model. The two significant departures from expectation were (1) cases where the first pulse fell during the first 4 hours following the LL/DD transition (Fig. 3.22, panel 1) and (2) when the first pulse fell at Ct 17.7 in the second cycle (not illustrated). The first of these anomalies will be considered later (Chapter 3, D).

Experimental evidence for the pacemaker-slave architecture of the circadian system controlling the eclosion rhythm will be considered in Chapter 6.

Despite the strong experimental evidence in favour of interacting A (pacemaker) and B (slave) oscillators in the *D. pseudoobscura* eclosion rhythm, Chandrashekar (1980) questioned the existence of a separate B-oscillator by using 12 hour high (30°) or low (10°) temperature pulses to perturb the rhythm free-running in darkness at 20°C. Results suggested that both light *and* temperature pulses caused phase shifts of what had been considered the A-oscillator leaving, in Chandrashekar's opinion, no room for a separate B system. It is possible, however, that these differences in opinion arise from a too rigid interpretation of the original model, in which light sensitivity was restricted to A and temperature sensitivity to B. If both the pacemaker and the slave were sensitive to light *and* temperature pulses, two such interacting systems could be present at the physiological level – for example, in the eclosion rhythm case, a pacemaker in the brain and a slave in a brain-dependent tissue secreting an 'eclosion hormone'. Until more is known about the physiology of complex circadian systems such as that regulating pupal eclosion, the robust and highly predictive model proposed by Pittendrigh and Bruce should be retained.

In two species, phase response curves have been followed over several circadian cycles, or even throughout development. In *D. pseudoobscura* responses to 15-minute light pulses remained remarkably consistent throughout larval (Zimmerman and Ives, 1971) and intra-pupal development (Pittendrigh, 1966), and the eclosion rhythm could be entrained by light-cycles throughout this period. Responses to very short pulses (20 seconds, blue light, 10 μWcm^{-2}), however, produced a low amplitude or 'weak' Type 1 response curve during the first circadian cycle following entry into darkness, but 'strong' or Type 0 responses in the second and third cycles (Winfrey, 1972a). This change was associated with a slow dark adaptation rather than a developmental change. In *Sarcophaga argyrostoma*, on the other hand, the phase-response curve for single 12-hour pulses of white light (240 $\mu\text{W cm}^{-2}$) declined from a 'strong' Type 0 curve during the first 2 to 3 days of larval development to a 'weak' Type 1 in mature third instar larvae (Saunders, 1978a, 1979b). Intra-pupal stages were virtually unresponsive to such light pulses (Fig. 3.23) although 12-hour high-temperature pulses were capable of shifting phase (Saunders, 1979b). Unlike *D. pseudoobscura* this change in PRC type was only partially associated with dark adaptation; most was probably associated with developmental changes in the photoreceptor or in its coupling to the circadian pacemaker. In the blow fly *Lucilia cuprina*, the oscillator controlling pupal eclosion also became markedly less sensitive

to light pulses after puparium formation (Smith, 1985), although *repeated* light-dark cycles given at this stage of development could initiate and entrain the rhythm.

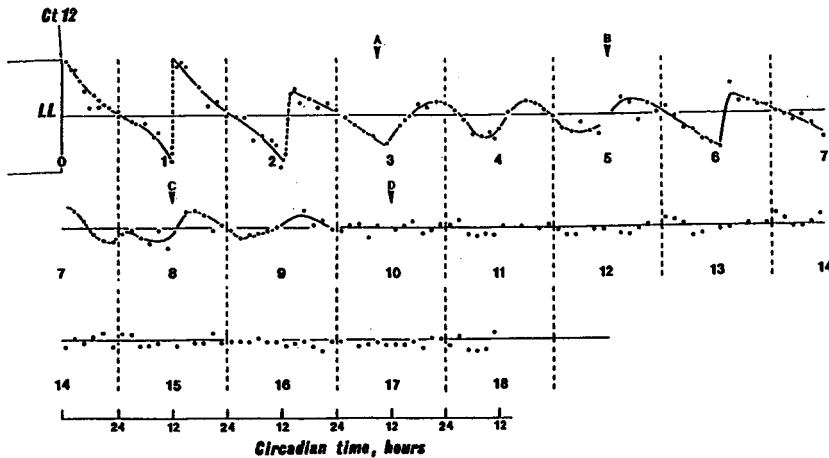


Fig. 3.23. Phase response curve for the eclosion rhythm in *Sarcophaga argyrostoma*: all stages of development exposed to single 12 hour pulses of white light ($240 \mu\text{W cm}^{-2}$) following transfer from LL to DD ($\approx \text{Ct } 12$) during the first 24 hours of larval life. Each plotted point shows the phase shift ($-\Delta\phi$ below the horizontal line; $+\Delta\phi$ above it) caused by the light pulse experienced by a batch of 200 to 700 larvae or pupae. A – larvae leave the meat; B – mature larvae sieved from sawdust; C to D – time of maximal puparium formation. Note how the PRC declines from a high ‘amplitude’ Type 0 (Winfree, 1970) to a low ‘amplitude’ Type 1 as the larvae mature, and is finally extinguished during the intra-puparial stages. (From Saunders, 1979b).

2. Entrainment by single recurrent light-pulses

The derivation of the phase-response curve for the pupal eclosion rhythm in *D. pseudoobscura* has been described above; its use in the prediction and interpretation of entrainment by light-cycles will now be examined.

When a self-sustained oscillation such as that controlling the eclosion rhythm (whose natural period is τ) is entrained by an external periodicity (whose period is T), the oscillation assumes the period of the driving cycle and maintains a fixed phase-relationship to it. This entrainment is effected by discrete and apparently instantaneous phase-shifts of the A-oscillator or pacemaker ($\tau - T = \Delta\phi_{ss}$) although, of course, there is usually a number of transient cycles before the driven system (B) controlling eclosion ‘catches up’. With recurrent 15-minute light pulses the interval between them defines the environmental cycle (T). For example, pulses 23 hours apart will define a 23-hour environmental period ($T = 23$), whereas pulses 27 hours apart will define $T = 27$. By effecting discrete phase-shifts the oscillation will become entrained to such cycles (Fig. 3.24).

Calculations of the approach to steady-state entrainment to *trains* of such pulses (and similar calculations for ‘skeleton’ photoperiods) are greatly facilitated by plotting PRC data in the form of phase-transition curves (Ottosen et al., unpublished), transformation curves (Johnsson and Karlsson, 1972b), or in the ‘old phase/new phase’ format of Winfree (1970). These are all roughly equivalent and are essentially plots of the circadian phase after the light pulse as a function of phase before the pulse. Particularly for longer light pulses, the

transformation method of Johnsson and Karlsson (1972b) is the most convenient because it incorporates pulse duration.

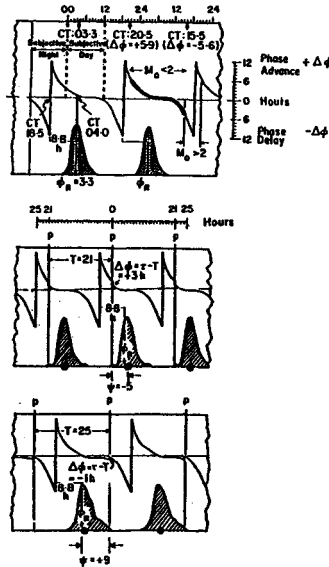


Fig. 3.24. The entrainment of the *D. pseudoobscura* oscillation by recurrent 15 minute light pulses. Upper panel: the phase response curve. Entrainment is only possible when the light pulse in the steady state falls at points on the PRC where its slope (M_0) is less than -2 (heavy line). Middle panel: entrainment by light cycles in which a 15 minute light pulse recurs every 21 hours ($T = 21$). In $T = 21$ the light pulse causes a phase advance of 3 hours in each cycle, and it falls at Ct 23.3 in the late subjective night. In $T = 25$ (lower panel) the light pulse causes a phase delay of 1 hour and falls on Ct 12.3 in the *early* subjective night. (From Pittendrigh, 1966).

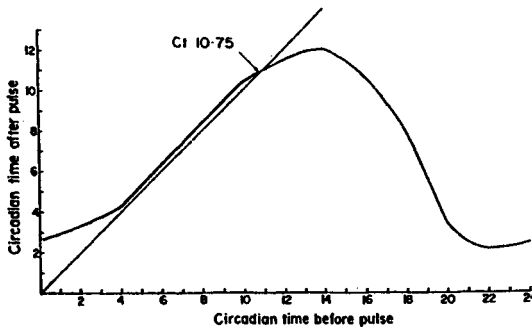


Fig. 3.25. *Drosophila pseudoobscura*. A plot of the circadian time of the eclosion rhythm after a 15 minute light pulse as a function of the circadian time before the pulse, according to the transformation method. The intersection between the transformation curve and the straight line drawn through the origin and with a slope of one, indicates the position of the 'fixed point'. A pulse of light commencing at the fixed point (Ct 10.75) theoretically leaves the oscillation at the *same* circadian time *after* the pulse, regardless of the duration of the light signal. (Redrawn from Johnsson and Karlsson, 1972b).

The transformation curve (Fig. 3.25) is calculated from the *D. pseudoobscura* standard PRC by the following method. A 15-minute pulse starting at Ct 0, for example, causes a discrete phase advance of +2.25 hours; the circadian time at the end of the pulse is therefore $0 + 2.25 + 0.25 = \text{Ct } 2.50$, because the duration of the pulse itself (0.25 hour) is added to account for the lapse of 'real' time. A pulse commencing at Ct 16, on the other hand, causes a delay of -6 hours, so that at the end of this pulse, the pacemaker is at $\text{Ct } 16 - 6 + 0.25 = \text{Ct } 10.25$. Similarly a pulse starting at Ct 22 ends at $22 + 3.85 + 0.25 = \text{Ct } 2.10$; in this case 24 hours is subtracted to keep the value within the circadian period. The transformation curve for strong Type 0 PRCs winds periodically along a horizontal line, whereas that for a weak Type 1 curve winds up with an average slope of 1.

Approaches to entrainment may be calculated by 'reading off' from the transformation curve the phase at the end of the pulse, for each pulse in the train as it occurs, or by arithmetic procedures (as above). As examples we can use *Zeitgeber* cycles of T 23 hours (LD 0.25:22.75) with the first pulse in the train starting at, say, Ct 22; and a cycle of T 27 hours (LD 0.25:26.75) with P_1 starting at Ct 16.

Example 1. T 23, P_1 starting at Ct 22

P_1 starts at Ct 22, transformed to $\text{Ct } 2.10 + 22.75$ (hours of darkness) = 24.85 - 24 = Ct 0.85

P_2 starts at Ct 0.85 $\rightarrow 2.84 + 22.75 = 25.69 - 24 = \text{Ct } 1.69$,

P_3 starts at Ct 1.69 $\rightarrow 3.14 + 22.75 = 25.99 - 24 = \text{Ct } 1.99$,

P_4 starts at Ct 1.99 $\rightarrow 3.25 + 22.75 = 26 - 24 = \text{Ct } 2.0$,

P_5 starts at Ct 2.0 $\rightarrow 3.25 + 22.75 = 26 - 24 = \text{Ct } 2.0$

Example 2. T 27, P_1 starting at Ct 16

P_1 starts at Ct 16 $\rightarrow 10.25 + 26.75 = 37 - 24 = \text{Ct } 13$,

P_2 starts at Ct 13 $\rightarrow 11.75 + 26.75 = 38.5 - 24 = \text{Ct } 14.5$,

P_3 starts at Ct 14.5 $\rightarrow 11.5 + 26.75 = 38.25 - 24 = \text{Ct } 14.25$,

P_4 starts at Ct 14.25 $\rightarrow 11.625 + 26.75 = 38.375 - 24 = \text{Ct } 14.375$,

P_5 starts at Ct 14.375 $\rightarrow 11.5625 + 26.75 = 38.3125 - 24 = \text{Ct } 14.3123$,

P_6 starts at Ct 14.3

In Example 1, the circadian oscillation underwent four or five transient cycles before reaching steady state; the pulse then came on at Ct 2 and ended at Ct 3.25. Application of similar calculations for other initial conditions (starting phases) would show that, for any *Zeitgeber* cycle of T 23, the light-pulse *always* comes to illuminate Ct 2 in steady state, *irrespective of the phase at which P_1 starts*, but after a different number of transient cycles. Inspection of the 'standard' PRC for 15-minute light-pulses (Fig. 3.17) will show that pulses falling at Ct 2 generate an advance of 1 hour, precisely the phase-shift required in each cycle to 'correct' τ (~24 hours) to T (23 hours). In Example 2, the oscillation underwent five or six transients before reaching steady state in which the pulse came on at about Ct 14.3. At this phase the pulse generates a phase *delay* of 3 hours, necessary to correct τ (~24 hours) to T (27 hours). Note also that in these two examples, the oscillation attains quite a different phase relationship (ψ) to the *Zeitgeber* cycle. In the first case, the pulse came on at Ct 2; therefore the median of pupal eclosion (at Ct 3) would coincide with the light. In the second case the pulse

came on at Ct 14.3, so that eclosion would occur about 11.3 hours *before* the light-pulse. In reaching steady-state entrainment to these two *Zeitgeber* cycles, therefore the two requirements - adjustment of period from τ to T and the adjustment of phase angle (ψ) - have both been achieved. Similar calculations for a range of T -values reveal two generalisations. First, when T is shorter than τ the oscillation phase-lags the driving light-cycle; consequently the light-pulse will fall in each cycle in the late subjective night or early subjective day to cause a phase advance ($+\Delta\phi$). Conversely, when $T > \tau$ the oscillation will phase-lead the driver, the pulse will fall in the early subjective night, and generate a phase-delay ($-\Delta\phi$) (see Fig. 6.11). The second point concerns the range of effective T -cycles. Computer simulations have shown that steady-state entrainment is only possible when the light-pulses fall on the response curve (Fig. 3.17) at points where the (negative) slope is less than -2.0 (Pittendrigh, 1966). The maximum utilisable phase advance is therefore $+5.9$ hours and the maximum utilisable phase delay is -5.6 hours; the primary *range of entrainment* is, therefore, from about 18 to 30 hours. In practice it is from about 19 to 29 hours. Outside this range, entrainment breaks down, but with still longer or shorter T -values the oscillation may lock on to multiples or submultiples of τ (see also Chapter 2, D. 1).

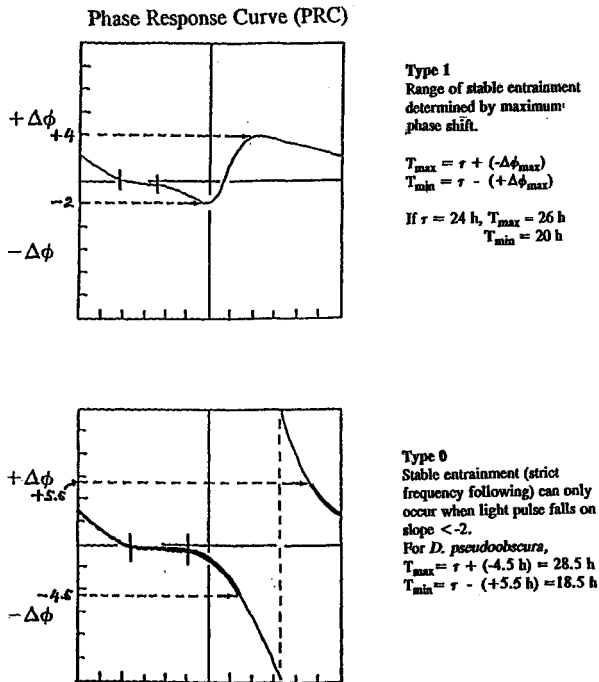


Fig. 3.26. How phase response curves predict the primary range of entrainment of a circadian oscillation. Upper panel: a 'weak' Type 1 PRC (low amplitude); range of entrainment is determined by the maximum delays and advances, in this (theoretical) case it is from about 20 to 26 hours. Lower panel: a 'strong' Type 0 PRC (high amplitude); here stable entrainment can only occur when the light pulse falls on the PRC where its slope is less than -2 . For *D. pseudoobscura* exposed to 15 minute light pulses, the range of entrainment is from about 18.5 to 28.5 hours. (Original).

Figure 3.26 summarises theoretical ranges of entrainment of a circadian oscillator exposed to 'weak' pulses (giving rise to a Type 1 PRC) or to 'strong' pulses (giving rise to a Type 0 PRC). In the former (upper panel), maximum sustainable advances and delays are determined by the maximum values of phase shift (+ and $-\Delta\phi$); the example given shows a range of entrainment to light cycles (T) of about 20 hours to 26 hours. For 'strong' pulses (lower panel) in which the PRC used is the 'standard' response of *D. pseudoobscura* to 15 minute light pulses (Fig. 3.17), stable entrainment can only occur when the light pulse falls on the slope when it is less than -2 (Pittendrigh, 1966). For the eclosion rhythm in *D. pseudoobscura*, this range of entrainment is from about 18.5 to 28.5 hours. Light pulses falling on the PRC where its slope is greater than -2 cannot yield a steady state.

Skopik and Takeda (1980) demonstrated similar entraining effects for 1 hour pulses of light interacting with the oviposition rhythm in the European corn borer, *Ostrinia nubilalis*. In a T = 19 hour cycle (LD 1:18), the light pulse came to illuminate, in steady state, a point in the late subjective night (\sim Ct 23) to generate the necessary phase advance, whereas in T = 26 hour (LD 1:25) the pulse came to lie in the early subjective night to generate the required phase delay. The range of entrainment to trains of 1 hour pulses was from about 19 to 26 hours.

Pittendrigh (1981a) confirmed that entrainment is effected by instantaneous phase-shifts by using the method of 2 pulses per cycle, described before. In this case, however, the first pulses were repeated to provide *Zeitgeber* cycles of T = 21 and T = 27 hours, and the second pulses were used to construct phase response curves to "describe the oscillation's time-course" (Fig. 3.27). The results were consistent with the non-parametric entrainment model. This model was also been applied, with success, to the eclosion rhythm in *Sarcophaga argyrostoma* (Saunders, 1978a) and for the calculation of entrained steady states within that species' photoperiodic clock (Chapter 11).

In a mathematical analysis of the *Drosophila* eclosion rhythm, Johnsson and Karlsson (1972b) drew attention to the fact that a line drawn through the origin and having a slope of one, intersected the transformation curve (Fig. 3.25) at a single point called the 'fixed point'. Evidently a pulse commencing at this phase (Ct 10.75) also leaves the oscillation at the end of the pulse at the same phase (i.e. $-\Delta\phi$ equals the duration of the pulse). Similar fixed points were derived theoretically for longer and shorter light pulses, the conclusion being that a light pulse starting at Ct 10.75 should always end at Ct 10.75, regardless of its duration, as though the driving oscillation had been 'frozen' during the time when the light was on. The fixed point has yet to be determined experimentally for *D. pseudoobscura*, but in *Sarcophaga argyrostoma* transformation curves for pulse durations of 1, 4, 8, 12 and 20 hours all nest together at a fixed point close to Ct 12 (Fig. 3.28). The significance of the fixed point is unclear, but it may be related to the observation that the circadian pacemaker is (for practical purposes) at or close to Ct 12 at the end of a long light-pulse, or at the LL/DD transition (see Chapter 3, D).

3. Entrainment by 'skeleton' photoperiods

Pittendrigh and Minis (1964) reported the important fact that two brief pulses of light *n* hours apart within each 24-hour cycle entrained the *D. pseudoobscura* rhythm to a steady-state phase-relation to such cycles that was identical to the phase-relation it assumed when entrained by a single long-duration pulse of *n* hours. They described the two pulses as a 'skeleton' photoperiod (PP_s) which simulated, in its action on the oscillator, the effect of a 'complete' photoperiod (PP_c). In the later chapters on photoperiodism (see Chapter 11), we will see further examples of how skeleton photoperiods may simulate the action of complete photoperiods in the induction of diapause. This phenomenon demonstrated the importance of the 'on' and 'off'

signals of the photoperiod in the entrainment and phase-control of oscillations; the time interval between them is also important, but the fact that there is, or is not, light between the pulses is in some cases immaterial.

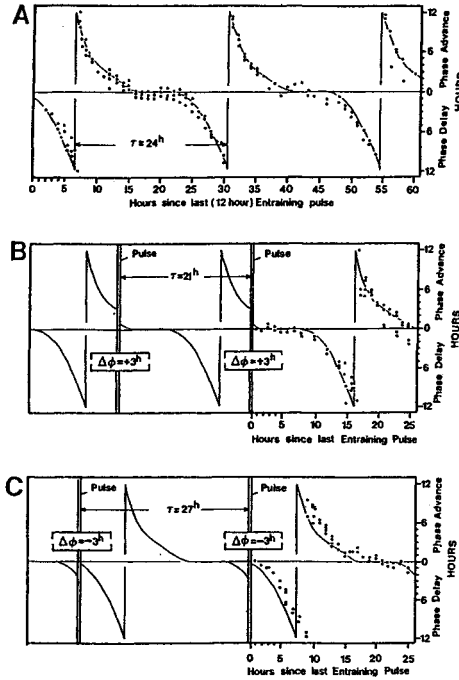


Fig. 3.27. *Drosophila pseudoobscura*, pupal eclosion rhythm: time course of the free running and entrained pacemaker assayed by a succession of phase shift ($\Delta\phi$) responses. A – the pacemaker free running for three cycles in DD after release from LD 12:12; B and C – plot the predicted pacemaker time course in relation to 15 minute light pulses recurring at 21 hour ($T = 21$) and 27 hour ($T = 27$) intervals. After release from entrainment by n cycles, the phase of the pacemaker is assayed (by the second pulse technique; see text) relative to the last seen entraining pulse. Predicted and assayed time courses are very close. (From Pittendrigh).

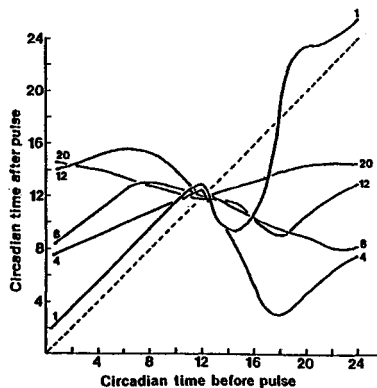


Fig. 3.28. Transformation curves for the eclosion rhythm in *Sarcophaga argyrostoma* (see also Fig. 3.25 and text), for light pulses ($240 \mu\text{W cm}^{-2}$) of 1, 4, 8, 12 and 20 hours duration. Note how all the curves 'nest' together close to Ct 12. (Original).

Pittendrigh and Minis (1964) described two types of skeleton photoperiod. 'Symmetrical' skeletons were defined by two pulses of equal duration (say 15 minutes each), whereas 'asymmetrical' skeletons were defined by a longer, or 'main', photoperiod (say 4 to 14 hours in length) and a secondary pulse scanning the 'night'. The entrainment of the oscillation by symmetrical skeletons will be considered first.

In the *D. pseudoobscura* case symmetrical skeleton photoperiods consisting of two 15-minute pulses of white light simulate almost perfectly the action of complete photoperiods (at $T = 24$ hours) up to about 11 hours (Fig. 3.29)(Pittendrigh, 1965). The phenomenon of entrainment by these two-point skeletons may be explained by the use of the phase-response curve for 15 minute pulses, and the non-parametric model outlined earlier, if the final steady-state phase is computed from a sum of the two phase-shifts caused by the two separate pulses.

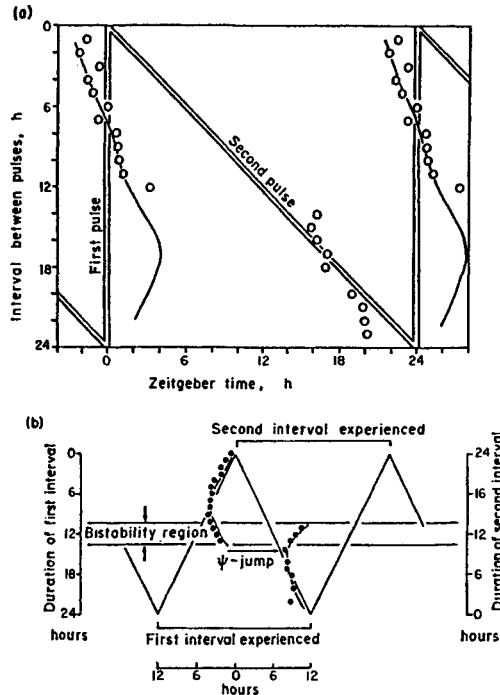


Fig. 3.29. (a) The phase of the *D. pseudoobscura* eclosion rhythm as a function of symmetrical skeleton photoperiods. The plotted points are medians of the steady state distributions of eclosion. The solid curve is that fitted to medians for complete photoperiods (see Fig. 3.1). Note the phase jump that occurs at about PP_s 14:10. (From Pittendrigh, 1965). (b) ditto, showing the 'region of bistability' (between PP_s 10.3 and PP_s 13.7) for which there are two possible steady states with radically different ψ -values. (From Pittendrigh, 1966).

Thus in steady state, when the oscillator assumes the period of the driving light-cycle (τ is changed to T), the following relation holds:

$$\tau - T = (\Delta\phi_1) + (\Delta\phi_2)$$

where $\Delta\phi_1$ is the phase-shift caused by one light-pulse (P_1) in the skeleton photoperiod and $\Delta\phi_2$ is the phase shift caused by the second pulse (P_2). Figure 3.30 shows how the phase-shifts derived from the PRC may be used to calculate the steady-state phase relationship by graphical means.

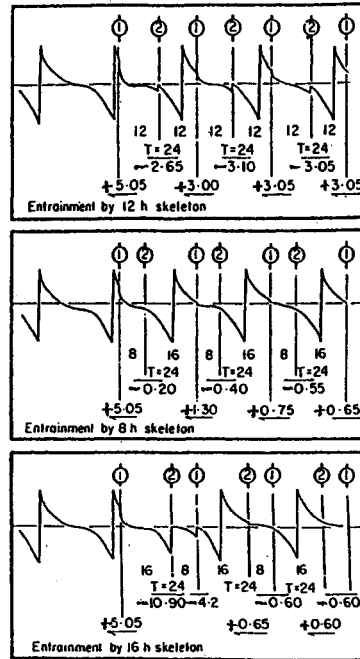


Fig. 3.30. The entrainment of the *D. pseudoobscura* oscillation by 2-pulse (symmetric) skeleton photoperiods, showing how the phase response curve may be used to predict the resetting phase of the eclosion rhythm. The panels show the $\Delta\phi$ s generated by the two 15 minute pulses (1 and 2) in PP_s 12:12 (upper) and PP_s 16:8 (lower). In the case of PP_s 16:8 (lower panel) the steady state phase of the oscillation eventually becomes identical to that of PP_s 8:16. (Redrawn from Pittendrigh, 1965).

Symmetrical skeleton photoperiods, however, differ from complete photoperiods in one important respect: there is no continuous light between the 'on' and 'off' signals. For this reason a skeleton can be interpreted in two ways. A skeleton of PP_s 10:14, for example, is also a skeleton of PP_s 14:10, the only difference between the two being one of phase. Which of the two interpretations the oscillator finally accepts depends on a number of factors, the most important of which is the duration of the interval between the two pulses. When the interval between the pulses is less than about 11 hours the simulation of PP_c by PP_s is almost perfect. With PP_s 12:12, simulation is less good in the sense that the phase relationship (ψ) of eclosion to the pulse acting as dawn is no longer the same as that under a complete photoperiod of 12 hours. With skeletons longer than 14 hours (PP_s 14:10 and over), however, the light fails completely to simulate a 14-hour photoperiod and the oscillator 'leaps' to the second 'interpretation', namely that of PP_s 10:14. In *D. pseudoobscura* the general rule is that when the two pulses have an interval between them of longer than 13.7 hours, the rhythm

always assumes a phase-relation which is characteristic of the *shorter* duration (Pittendrigh, 1966). The change in phase is called a 'phase-jump' (ψ -jump) (Fig. 3.30).

Skeleton photoperiods close to $\tau/2$ show an additional property first revealed by computer simulation (Pittendrigh, 1966). All such cycles between PP_s 10.3 and PP_s 13.7 are open to two distinct interpretations, and the steady state ultimately adopted depends on two variables: (1) the phase (circadian time) which the *first* pulse illuminates and (2) the value of the first *interval*. Figure 3.31 compares the positions of the eclosion peaks adopted in four skeleton photoperiods: PP_s 9:15, PP_s 15:9, PP_s 11:13 and PP_s 13:11. For the first two regimes the resulting steady states are unique and the phase angle (ψ) finally adopted is always that of the shorter interval (ψ_9 in both). For the other two, however (both of which lie in the zone from PP_s 10.3 to PP_s 13.7), the phase adopted depends on the circadian time at which the first pulse is seen. For example, a PP_s 11:13 assumes the phase characteristic of PP_s 11:13 (ψ_{11}) when the first interval seen is 11 hours and the first pulse falls at Ct 03 or Ct 22, but assumes a phase characteristic of PP_s 13:11 (ψ_{13}) if the first pulse falls at Ct 11 or Ct 16. Conversely, a PP_s 13:11 assumes ψ_{13} when the first interval seen is 13 hours and the first pulse falls at Ct 03 or Ct 22, but assumes ψ_{11} if the first pulse falls at Ct 11 or Ct 16. This region close to $\tau/2$ is called the 'zone of bistability' (Pittendrigh, 1966). Exactly comparable phenomena have been described for the eclosion rhythm in *D. melanogaster* (Ottesen et al., unpublished), the oviposition rhythm in the European corn borer *Ostrinia nubilalis* (Skopik and Takeda, 1980) and in the eclosion rhythm of the flesh fly *Sarcophaga argyrostoma* (Saunders, 1978). In later sections (Chapter 11) we will see similar behaviour of the oscillators involved in photoperiodic induction.

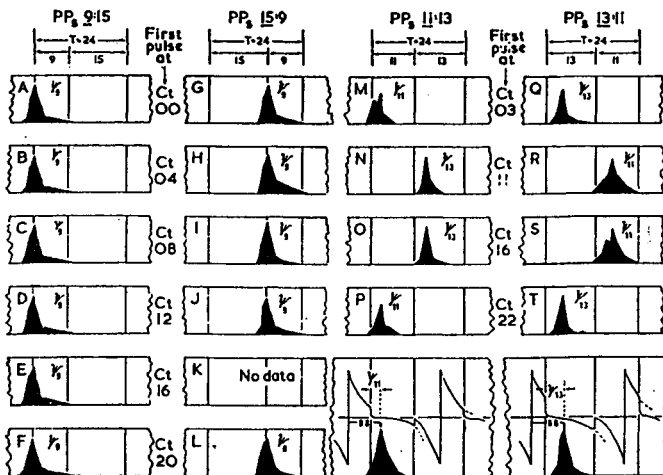


Fig. 3.31. The 'bistability' phenomenon in the *D. pseudoobscura* eclosion rhythm. A unique phase relationship (ψ_9) between the rhythm and the light cycle develops for PP_s 9:15 and PP_s 15:9, no matter where the first pulse that initiates the entraining cycle falls on the Ct scale. On the other hand, for both PP_s 11:13 and PP_s 13:11 there are two alternative steady states, ψ_{11} and ψ_{13} . Which develops depends on where the first pulse that initiates the entraining cycle falls on the Ct scale. The steady state of ψ_{11} and ψ_{13} are shown in terms of the phase response curve in the two lower right-hand panels. (Redrawn from Pittendrigh, 1966).

Asymmetrical skeleton photoperiods also show the phenomenon of a phase-jump. Figure 3.32 shows the steady-state phase of the eclosion rhythm in *D. pseudoobscura* in asymmetrical ($T = 24$ hour) skeletons comprising a 4-hour main photoperiod and a 15-minute pulse scanning the accompanying dark period. As the 'night interruption' falls later and later, from Zt 05 to Zt 12, the phase of the rhythm moves to the right. When the pulse falls at Zt 14 and later, however, a ψ -jump occurs and the peak of eclosion moves to later clock hours thus accepting the shorter interval between the two pulses. It is clear that when the pulse falls early in the night it is 'read' as a terminator of a skeleton photoperiod (a 'new dusk'), but when the pulse falls late in the night it is read as an initiator (a 'new dawn'). With longer and longer main photoperiods the position of the ψ -jump becomes later and later. In all cases except those close to the ψ -jump, simulation of the corresponding complete photoperiod is good. Similar effects have been described for the action of asymmetric skeleton photoperiods on the oviposition rhythm in *Pectinophora gossypiella* (Pittendrigh and Minis, 1964, 1965), on the pupal eclosion rhythm in the same species (Pittendrigh and Minis, 1971), and in *Sarcophaga argyrostoma* (Saunders, 1978a). Asymmetric skeleton photoperiods are of particular interest in the analysis of the photoperiodic clock and will be examined further in Chapter 11.

Drosophila pseudoobscura Pupal Eclosion Rhythm 20°C

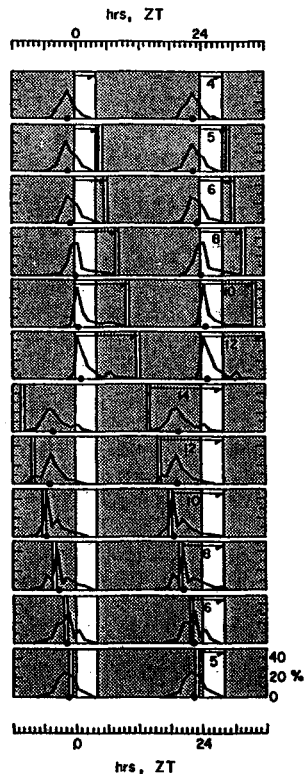


Fig. 3.32. The entrainment of the *D. pseudoobscura* eclosion rhythm by asymmetric skeleton photoperiods comprising a main photoperiod of 4 hours and 15 minute night interruptions. The effective skeleton is indicated by arrows and its duration by the number (of hours) below it. Note the phase jump when the skeleton exceeds about 14 hours. (From Pittendrigh, 1965).

The importance of both 'on' and 'off' signals of the photoperiod in entrainment and phase control was recognised by Pittendrigh (1960, 1965) and evident, for example, in the simulation of complete photoperiods by skeleton regimes. Several authors have since attempted to separate the effects of these signals by examining light-steps as opposed to light-pulses. Engelmann (1966) attempted to explain the eclosion rhythm in terms of 'on' and 'off' oscillators. Chandrashekar (1967b) transferred populations of *D. pseudoobscura* from LD 12:12 to DD and then exposed the cultures to either a single 15-minute light-pulse or to a light-step (light 'on' without light 'off') at Ct 15.5 or at Ct 21.5. It was found that the pulses caused, as expected, a $-\Delta\phi$ of 5.0 hours at Ct 15.5 and a $+\Delta\phi$ of 4.4 hours at Ct 21.5. The light steps, however, caused an appreciable advance in phase at both circadian times. Chandrashekar postulated that the light 'off' signal in the early part of the subjective night acted as a 'new dusk' and was effective in causing a delay phase-shift, whereas the light 'on' signal in the second half of the night was interpreted as a 'new dawn' and caused a phase advance. The light step, however, had no 'off' component and only produced its effect (phase advance) when light extended into the second half of the night. A simpler alternative, however, might be that continuous light merely caused τ to shorten (see, for example, Bruce and Pittendrigh, 1957; Chandrashekar and Loher, 1969b).

In a later paper, Chandrashekar et al. (1973) systematically scanned the subjective night with light pulses of between 15 minutes and 6 hours duration. These pulses were arranged in four groups. In the first half of the night (Ct 12 to 18) there were two groups with either 'on' signals or 'off' signals synchronised respectively; in the second half of the night (Ct 19 to 01) the other two groups were similarly arranged. The results showed that the 'off' transitions determined both the direction and the magnitude of $\Delta\phi$ during the first part of the night, whereas the 'on' transitions determined $\Delta\phi$ in the latter half. Similar effects were noted when phase-response curves for pulses between 15 minutes and 12 hours were plotted for either the 'on' or the 'off' transitions. The 'off' signal in the first half of the night clearly simulated a 'new dusk' and caused a delay phase-shift. The 'on' signal in the second half of the night, however, simulated a 'new dawn' and caused an advance phase-shift, as in the asymmetrical skeleton photoperiods first described by Pittendrigh and Minis (1964).

D. EFFECTS OF CONTINUOUS LIGHT AND EXTENDED LIGHT PERIODS

Early investigations of the effect of LL on the eclosion rhythm in *D. pseudoobscura* (Pittendrigh and Bruce, 1957; Chandrashekar and Loher, 1969b) showed a fairly rapid 'damping out' to eventual arrhythmicity, the number of persistent cycles depending upon intensity. Winfree (1974) later made a systematic study of the effects of low intensity blue light. Below about $0.001 \text{ erg cm}^{-2} \text{ sec}^{-1}$ the rhythm persisted, adults emerging in 6-hour gates roughly 24.7 hours apart. Above about $0.1 \text{ erg cm}^{-2} \text{ sec}^{-1}$, however, adults emerged at random. Between these two values the emergence peaks became progressively broader, the first trends towards arrhythmicity becoming apparent after about 7 days in a light intensity of $0.01 \text{ erg cm}^{-2} \text{ sec}^{-1}$. Winfree likened the lower limit to that of 'starlight' and the upper limit to 'moonlight': clearly the overt rhythm of pupal eclosion in *D. pseudoobscura* is eliminated at very low light intensities, much lower, for example, than most of the behavioural rhythms reviewed in Chapter 2.

The apparent dampening effect of continuous light on 'population' rhythms varies between species. Whereas *D. pseudoobscura* appears to be exquisitely sensitive to low light intensities, the egg hatching, pupation and eclosion rhythms of the southwestern corn borer, *Diatraea grandiosella* (Takeda, 1983), the egg hatching rhythm of the cricket, *Gryllus*

bimaculatus (Tomioka et al., 1991) and the eclosion rhythm of the blow fly, *Lucilia cuprina* (Smith, 1985) all persist for at least a few cycles in LL of much higher intensity.

It has been known for a long time that, although eclosion in *D. pseudoobscura* may become arrhythmic in LL, a rhythm becomes apparent again after a transfer to darkness, with peaks of eclosion occurring about 15 + modulo τ hours after the LL/DD transition. This observation suggests that the oscillation is damped out in LL, but restarts at a particular phase (called Circadian time, Ct 12) upon transfer to darkness (Chapter 3, C. 1).

The minimum length of light necessary to cause this damping has been investigated by Pittendrigh (1960, 1966). Eighteen populations of *D. pseudoobscura* were raised in LD 12:12 and then exposed to a last photoperiod of varying duration before release into DD (Fig. 3.33). The intensity of the light was about 100 f.c. Dawn of the last photoperiod came at its previously established phase, but dusk was either advanced or delayed, relative to the control, by various amounts in the several cultures. The results showed that after a terminal photoperiod of 12 hours or more the steady-state phase-points of the eclosion peaks occurred at fixed time intervals ($n\tau + 15$ hours) after the LL/DD transition, suggesting once again that the driving oscillation stops in protracted light.

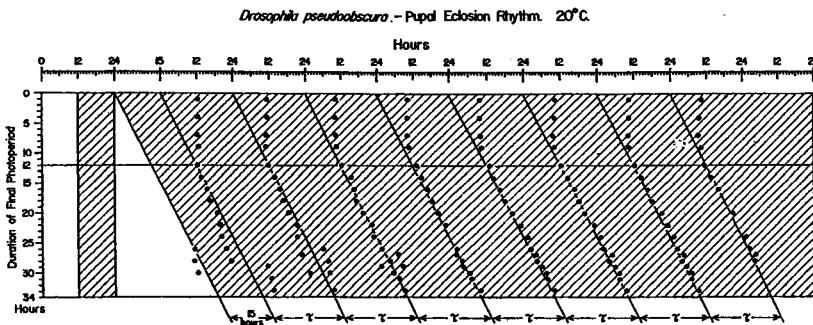


Fig. 3.33. The effect of varying the duration of the final photoperiod prior to releasing cultures of *D. pseudoobscura* into DD free run. After a final photophase longer than 12 hours the steady state phase of the rhythm obtains its principal 'time cue' from dusk, or light off; the inference is that the driving oscillation begins afresh from Ct 12 at the final light/dark transition. (From Pittendrigh, 1966).

A systematic study of the $\Delta\phi$ s generated by 15-minute light-pulses provided a phase-response curve for the oscillation during the hours immediately following the LL/DD transition; this proved to be identical to that following LD 12:12 (Fig. 3.34). The implication of this result is that photoperiods longer than 12 hours not only damp out the oscillation, but hold the oscillations (in each fly in the population) in the same fixed state which corresponds to that at Ct 12, so that, on entry or re-entry into darkness, all the oscillations in the population resume their motion at the same phase. A further implication, which is especially important in the interpretation of some photoperiodic phenomena (Chapter 11), is that with photoperiods in excess of 12 hours, the principal (or only) time signal of significance is that associated with 'dusk'; with shorter photoperiods, however, both dawn and dusk play a role in phase-control. The oscillation controlling adult eclosion in the flesh fly, *Sarcophaga argyrostoma*, is similarly damped out by photoperiods in excess of 11 to 12 hours (Saunders, 1976, 1978a).

There are, however, several interesting complications to this apparently simple story. These suggest that the circadian pacemaker (A), as opposed to the driven rhythm or 'slave' (B),

may not be damped out by quite high light intensity, and that the pacemaker may not be reinitiated, at Ct 12, at the LL/DD transfer. The first complication was revealed in the two-pulse experiments described earlier (Chapter 3, C. 1)(Pittendrigh, 1974; 1981a), designed to investigate the apparently instantaneous resetting of the pacemaker by short light-pulses (see Fig. 3.22). In these experiments, anomalous results were obtained when the first pulse started during the first 4 hours of darkness following the LL/DD transition (defined as Ct 12 to 16). The subsequent characterisation of the oscillation's time course by the series of second pulses produced phase shifts several hours less than predicted. The interpretation of this anomaly is that although the pacemaker may be at a phase equivalent to Ct 16 4 hours after LL/DD, it is not necessarily at Ct 12 at the transfer itself (although for many practical purposes it may be regarded as being so). The second observation is that the pacemaker is also 'at Ct 12' when the flies are transferred to darkness from lower light intensities, even from those in which the overt rhythm clearly persists. Perhaps the interpretation of this unresolved issue is that, although the overt rhythm of eclosion may be damped out in LL, constant light of quite high intensity does not suppress the motion of the underlying pacemaker (although it may reduce its amplitude), and that the latter is reset to a phase equivalent to Ct 12 at the LL/DD transition, and certainly to Ct 16 4 hours later.

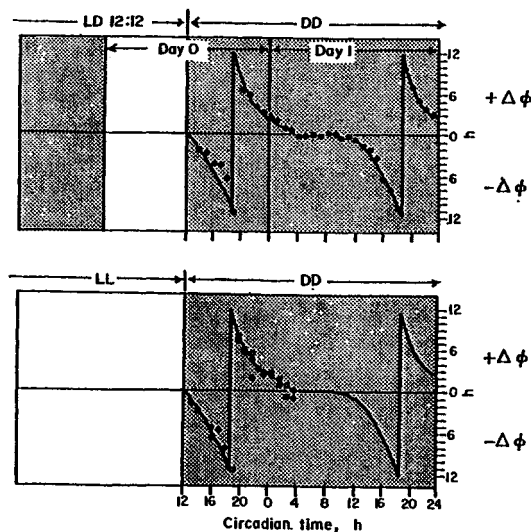


Fig. 3.34. Phase response curves for the *D. pseudoobscura* eclosion rhythm in DD free run after release from LD 12:12 (top panel) or from LL (lower panel), suggesting that the oscillation restarts at Ct 12 after light periods in excess of 12 hours. (From Pittendrigh, 1966).

Continuous light of higher intensities ($240 \mu\text{W cm}^{-2}$) also fails to stop the eclosion clock in *Sarcophaga argyrostoma*, although it may broaden the eclosion peaks (Saunders, 1979b); and the pacemaker, as in *D. pseudoobscura*, is apparently reset to a phase equivalent to Ct 12 upon transfer to darkness. Evidence for this proposition comes from an experiment similar in design to that described in Fig. 3.35, but with somewhat different results. Cultures of *S. argyrostoma* larvae were transferred from LL into a dark period of 12 hours, and then into a photoperiod of varying duration before final entry into DD. In all regimes in which the last

light period exceeded 12 hours, the peaks of eclosion (determined as medians of the gates) occurred at a characteristic time ($11.6 + \text{modulo } \tau$ hours) after lights-off. However, when the hours of darkness (D) and the terminal light period (L) added up to a value (D + L) of 24, 48, 72 or 96 hours (i.e. modulo τ) the peaks of eclosion were sharp and coherent, whereas when D + L added up to a value of 36, 60, 84 or 120 hours (i.e. modulo $\tau + \frac{1}{2}\tau$) the eclosion patterns were virtually arrhythmic (Fig. 3.35). One interpretation of this 'resonance effect' is that extended light periods failed to stop the motion of at least some of the oscillators in the population, and that the transition from light to dark was insufficiently 'strong' to reset all of them to the unique phase (equivalent to Ct 12) upon entry into darkness. One thing, however, is clear from these complex results with *D. pseudoobscura* and *S. argyrostoma*: that is, although constant light of various intensities may apparently 'damp out' the overt rhythm of pupal eclosion, it may not stop the underlying circadian pacemaker. This may continue to oscillate in LL, although with a reduced amplitude, and is reset to a characteristic phase within a few hours of its return to darkness.

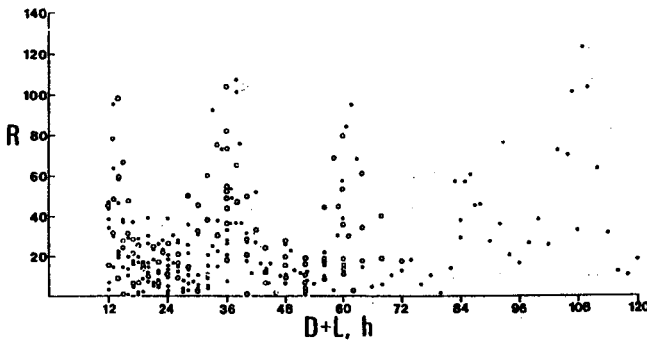


Fig. 3.35. *Sarcophaga argyrostoma*, eclosion rhythm. Arrhythmicity (Winfree's 1970 R values) of reset cultures as a function of D + L (D = hours of darkness between the LL/DD transition and the start of the perturbing light pulse; L = duration of light pulse). When D + L is close to 12, 36, 60 or 84 hours (modulo $\tau + \frac{1}{2}\tau$) arrhythmicity is high; when D + L is close to 24, 48, 72, 96 or 120 hours (modulo τ) it is low. Open circles – L > D; closed circles – D > L. (From Saunders, 1978a).

Similar interpretations have been applied to data for the flight-activity rhythm of the mosquito *Culex pipiens quinquefasciatus* (Peterson and Jones, 1979; Peterson, 1980 a, b) (and for eclosion in *S. argyrostoma* (Peterson and Saunders, 1980)). Using a simple limit cycle interpretation of the circadian oscillation (Pavlidis, 1968), the pacemaker's motion in LL was seen as one of reduced amplitude either outside the dark limit cycle (in *Culex*) or within it (*Sarcophaga*), the amplitude of the light limit cycle and its displacement from the dark limit cycle depending on light intensity and/or photoreceptor sensitivity. At even quite high light intensities the pacemakers in these species are thought to remain in motion, and the limit cycle model may be used to explain many features including 'resonance' effects on phase and eclosion coherence (Peterson and Jones, 1979; Saunders, 1978a) consequent upon a release to darkness after different lengths of time in the light. (For the limit cycle and mathematical treatments of these oscillations, the reader is referred to Peterson's papers; see also Chapter 7).

E. THE 'SINGULARITY POINT'

Winfree (1970 a, b) showed that the oscillation controlling the eclosion rhythm in *D. pseudoobscura* could be 'abolished' or placed in a completely 'phase-less' state by a single short light signal provided that signal was applied precisely at the right moment. Populations of flies placed in this 'phase-less' state showed an arrhythmic eclosion pattern as though the clock had been stopped. The critical signal necessary to stop the clock is referred to as the 'singularity'. Theoretical aspects of this singular point are considered in Winfree's papers; here we will confine our observations to the phenomenon in biological terms.

Mixed-age populations of *D. pseudoobscura* were raised in LL and then released into DD during intra-pupal development, this LL/DD transfer resulting, as we have seen earlier, in the generation of a clear rhythm of eclosion with peaks at circadian intervals. A variable time (T) after the LL/DD transfer pulses (S) of dim blue light ($10 \mu\text{W cm}^{-2}$) of less than 3 minutes' duration were applied to the cultures, and the eclosion peaks then recorded 3 days later after any transients had subsided. Values of T and S were varied systematically, and the interval between the end of the light perturbation (S) and the centroid of the eclosion peaks (called the cophase, θ) were plotted for each combination of T and S. When all the cophase data for a number of days were plotted in this manner the 'cloud' of dots gave a helicoid or spiral ramp, described by Winfree as a "vertical corkscrew linking together tilted planes" (Fig. 3.36).

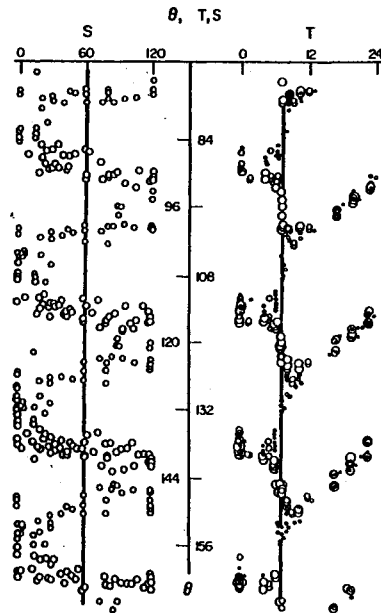


Fig. 3.36. *Drosophila pseudoobscura*. The cophase surface, $\theta(T, S)$, shown in two orthogonal projections. On the left the projection shows points $T < 7.3$ hours as larger circles and $T > 7.3$ hours as smaller circles. T from hour 15 to 24 is deleted, since this portion of the surface forms a tilted plane which would obscure the spiral ramp between $T = 0$ and $T = 15$ hours. The right hand projection shows data at $S = 0$ to 30 seconds as dots, 31 to 60 seconds as tiny circles, 61 to 90 seconds as larger ones, and the largest circles represent peaks of emergence following perturbations longer than 90 seconds. (From Winfree, 1970b).

This complex surface can also be depicted in two dimensions by plotting θ -contours above the $T \times S$ plane (Fig. 3.37). These contours represent the time after the stimulus (S) at which emergence peaks occur, plus multiples of 24 hours. The most interesting feature of this figure is that the cophase contours are confluent at a point (T^*S^* , or the singularity) at which the circadian rhythm is 'abolished' or put into a completely 'phase-less' state. In the spiral ramp depicted in Fig. 3.36 the symmetry axis corresponds to this singularity. Extensive experiments in which T and S were varied independently have shown the singularity to be a 50-second pulse placed 6.8 hours after the LL/DD transition. The critical pulse (S^*) was therefore exactly intermediate in 'strength' between those (<50 seconds) giving rise to weak or Type 1 response curves and those (>50 seconds) giving rise to strong or Type 0 curves (Chapter 3, C.1). It is also of interest that the critical time (T^*) of 6.8 hours places the pulse of light close to the point of maximum phase shift in the 'standard' PRC (Fig. 3.17); that is, close to Ct 18.

The degree of 'coherence' of the eclosion peaks was measured in terms of arrhythmicity or R-values, calculated as the number of eclosions outside an arbitrary 8-hour gate, divided by the number of eclosions within that gate, and multiplied by 100 (Winfree, 1970a). R-values of less than 30 were considered to be 'highly rhythmic', and those over 90 as 'arrhythmic'. On statistical grounds completely arrhythmic cultures would show R-values approaching 130 to 150. In Winfree's experiments all eclosion peaks reset by conditions far from T^*S^* showed a high degree of rhythmicity (low R-values). As the values of T and S approached the singularity, however, there was a progressive broadening of the peaks and, at the singularity itself, eclosion became virtually arrhythmic (Fig. 3.38).

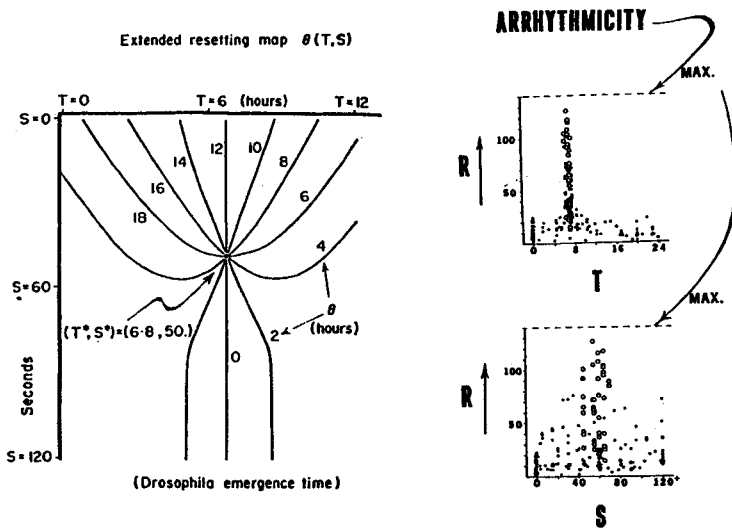


Fig. 3.37 (left). *Drosophila pseudoobscura*. Contour map of the cophase surface, $\theta(T, S)$, showing the critical annihilating pulse (T^*S^*) or the singularity that puts the clock into a non-oscillatory or 'phase-less' state. (From Winfree, 1970a).

Fig. 3.38 (right). *Drosophila pseudoobscura*, eclosion rhythm. Arrhythmicity values of reset cultures (see text for details) following a single pulse of light (duration 5 seconds) falling T hours after transfer from LL to DD. Arrhythmicity is highest when $T \sim 7$ hours and $S \sim 50$ seconds. This combination represents the 'singular stimulus' (From Winfree, 1970).

These results were taken as evidence for a critical annihilating light-pulse (T^*S^*) which puts the clock into a non-oscillatory state, i.e. "stops the clock". However, since the singular point is close to the point of maximum phase shift (at Ct 18), two interpretations are theoretically possible. In one, already outlined, the motion of the pacemaker is abolished; in the second the progressive broadening of the peaks and the final arrhythmicity represents nothing more than the mutual de-synchronisation of all the oscillators in the population. Winfree (1970b) recognised these alternatives and distinguished between them by giving a second perturbation of 120 seconds (pulse $S > S^*$) at different times after the singularity. Computer simulations suggested that if the arrhythmicity following perturbation at the singularity was due to a de-synchronisation of the constituent oscillators, the resulting rhythm of eclosion would apparently be markedly bimodal, whereas a re-initiation of a population of oscillators 'frozen' at the same phase would produce clearly unimodal eclosion peaks after the second perturbation. The experimental production of a unimodal distribution of phases suggested that re-initiation rather than re-synchronisation had occurred. The arrhythmicity of eclosion after T^*S^* thus appears to be equivalent to the 'primary' arrhythmicity of cultures raised from the egg in DD, with all of the oscillators in the population at rest (Chapter 3, A. 1) (Zimmerman, 1969).

A singular stimulus evoking arrhythmicity, and presumably 'stopping the clock', has since been observed in a number of organisms and oscillations. These include flower opening and closing in the plant *Kalanchoë blossfeldiana* (Engelmann and Johnsson, 1978), pupal eclosion in a short-period mutant (see Chapter 4) of *Drosophila melanogaster* (Winfree and Gordon, 1977) and in the eclosion rhythm of *Sarcophaga argyrostoma* (Saunders, 1978a). It has also been identified in a non-circadian system: the glycolytic oscillation in yeast (Winfree, 1973a). In *S. argyrostoma*, the singularity was reported to occur with a 4-hour pulse of white light ($240 \mu\text{W cm}^{-2}$) timed to commence 4 hours after the LL/DD transition; with this combination the normally rhythmic eclosion peaks adopted a markedly arrhythmic pattern (Saunders, 1978a). Using a simple graphical method to predict the clock's singularity, however, Vaz Nunes (1981) found the critical stimulus in *S. argyrostoma* to be closer to 3 hours.

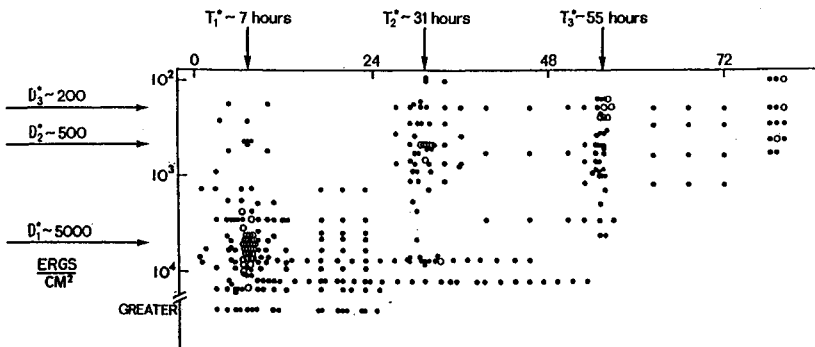


Fig. 3.39. *Drosophila pseudoobscura*, eclosion rhythm. Singular stimuli (T^*S^*) in the first three cycles following release from LL into DD. $T_1^*S_1^*$ is about 50 second pulse at about 7 hours; $T_2^*S_2^*$ is about 5 seconds at about 31 hours; $T_3^*S_3^*$ is about 2 seconds at 55 hours. Although T^* occurs at 24 hour intervals (modulo τ), S^* appears to be subject to 'dark adaptation'. Solid circles - $R < 30$; triangles - $60 > R > 30$; open circles - $R > 60$; R - arrhythmicity values. (from Winfree, 1972a).

Winfree (1972a) investigated the singularities for *D. pseudoobscura* in each of the first three cycles in DD and, surprisingly, found them to depart from a strict periodicity. For example, whilst S* in the first cycle was a 50-seconds pulse (of blue light, $10 \mu\text{W cm}^{-2}$), in the second cycle it was ~ 5 seconds, and in the third ~ 2 seconds (Fig. 3.39). This more than 10-fold increase in sensitivity over 3 days in DD was attributed to a slow 'dark adaptation'. In the short-period mutant of *D. melanogaster* (*per^S*, $\tau \sim 19$ hours; see Chapter 4), the singularities occurred about 19 hours apart as expected, but photosensitivity did not increase substantially with time in DD (Winfree and Gordon, 1977).

Theoretical considerations of the circadian oscillation in *D. pseudoobscura* suggested that the amplitude of the pacemaker declined as the singularity was approached (and at the singular point was completely damped). To test this, Winfree (1973b) carried out systematic two-pulse experiments in which the first pulses were placed at or close to the singularity, and the second pulses were used to measure its 'amplitude'. At this juncture it is sufficient to note that the circadian pacemaker was attenuated to zero amplitude after T*S*: these experiments and their interpretation are fully treated in Winfree's papers and in his book *The Geometry of Biological Time* (Winfree, 1980).

Chandrasekaran and Engelmann (1976) performed extensive experiments with the singularity in *D. pseudoobscura* in which they systematically varied the irradiance of the pulse (blue light) and its duration, but kept the onset of the pulse at 6.9 hours after LL/DD (Winfree's T*). It was found that the range over which reciprocity occurred was vast: pulses of 0.04 second duration and of $12,500 \mu\text{W cm}^{-2}$ were as effective in placing the oscillation on its singularity as were pulses of 50,000 seconds (13.9 hours) at $0.01 \mu\text{W cm}^{-2}$. The product, or critical radiant exposure, for all combinations, however, was close to $500 \mu\text{W cm}^{-2} \text{ sec}^{-1}$.

F. EFFECTS OF LOW OXYGEN TENSION AND COLD TORPOR

Kalmus (1935) and Pittendrigh (1954) showed that the clock controlling eclosion in *Drosophila* could be 'stopped' by hypoxia. Pittendrigh (1954), for example, subjected cultures of *D. pseudoobscura* to nitrogen containing traces of oxygen for a 15-hour period. On returning to aerobic conditions the first peak of eclosion was delayed by 15 hours, but the second and subsequent peaks by only 10. The similarities between this result and that for a temperature step (Chapter 3, A. 1) suggest that the first effect was possibly due to an effect on the B-oscillator, whilst the maintained phase-shift was the result of 'stopping' the basic A-oscillator.

In a follow-up of these experiments, Pittendrigh (1974) raised populations of *D. pseudoobscura* in LD 12:12 before releasing them into DD free-run. One set (of 24 cultures) was then placed in pure nitrogen for 1 to 24 hours, with the onset of anoxia commencing at the transfer from light to darkness (i.e. at Ct 12). A second set (of 36 cultures) was similarly treated, but with anoxia commencing 8 hours after the start of DD (i.e. at Ct 20). It was found that a successively longer exposure to N₂ starting in the early subjective night (Ct 12) caused a phase delay in the steady-state eclosion peaks, equal in magnitude to the time in N₂. When the nitrogen treatment commenced at Ct 20, however, the circadian pacemakers underwent a series of complex transients; they appeared to continue, or even accelerate, until the pacemaker reached the beginning of its oxygen-dependent phase when it stopped (Fig. 3.40). The results indicated that the circadian pacemaker has an O₂-dependent, energy-absorbing, or 'charge' phase in the early subjective night, perhaps lasting about 6 hours, and an O₂-independent, energy-dissipating, or 'discharge' phase in the late subjective night. Despite the fact that the concrete nature of circadian rhythmicity is not fully resolved, despite considerable progress at

the molecular level (see Chapter 4), these results must surely reflect real aspects of the physiology of its pacemaker.

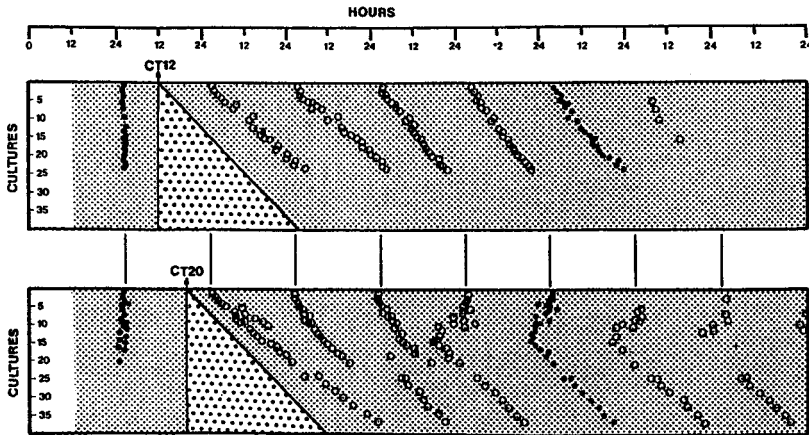


Fig. 3.40. The effect of anoxia (N_2) on the circadian oscillation gating pupal eclosion in *Drosophila pseudoobscura*. Upper panel: 24 populations transferred from LD 12:12 to DD with different cultures experiencing a period in pure N_2 (1 to 24 hours) commencing at the onset of darkness (Ct 12). Lower panel: 36 cultures similarly treated but exposed to periods of N_2 (1 to 36 hours) commencing 8 hours after the onset of darkness (Ct 20). Open circles – phase of rhythm after treatment; closed circles – steady state phases 5 days after treatment. Area with large stipples shows period of anoxia. See text for details. (From Pittendrigh, 1974).

Although τ for the eclosion rhythm in *D. pseudoobscura* is temperature-compensated, it is probable that cold torpor at temperatures approaching zero would effectively stop the motion of the oscillator. Apparently this effect has not been reported for the *D. pseudoobscura* case, but is well documented in other animals (Harker, 1958) and in plants.

B. EFFECTS OF TEMPERATURE PULSES AND CYCLES

1. Entrainment

Although the period of the oscillation controlling pupal eclosion in *D. pseudoobscura* is almost the same over a wide range of constant temperatures ($Q_{10} = 1.02$), temperature changes (e.g. steps, pulses and cycles) will cause phase-shifts and entrain (Pittendrigh, 1954, 1960). Temperature cycles will also entrain the eclosion rhythms of the fruit fly *Dacus tryoni* (Bateman, 1955), the moth *Anagasta kühniella* (Scott, 1936; Moriarty, 1959) and the egg hatching rhythm in the tettigoniid *Homorocoryphus jezoensis* (Arai, 1998).

Working with *Drosophila pseudoobscura*, Zimmerman et al. (1968) showed that a square-wave temperature cycle, or *thermoperiod*, consisting of 12 hours at 28°C and 12 hours at 20°C ($T = 24$ hours) will entrain the eclosion rhythm in DD. They also showed that single, non-recurrent, temperature pulses and temperature steps (up or down) will cause $\Delta\phi$ s similar in principle to those generated by light. The use of temperature steps is particularly interesting since the effects of a sharp rise and a sharp fall in temperature defining a pulse can be separated in a way which cannot be used for light 'on' and light 'off', because light 'on' (to LL) often leads to arrhythmicity (see section D) in which phase has no real meaning.

Zimmerman et al. (1968) showed that temperature steps-up from 20° to 28°C caused phase advances ($+\Delta\phi$), the magnitude of which depended on the phase of the oscillator exposed to the temperature signal. Figure 3.41 shows that the maximum $+\Delta\phi$ was obtained when the step-up occurred at about Ct 22. Conversely, temperature steps-down caused phase-delays ($-\Delta\phi$); once again the magnitude of the response depended on phase. The amplitude of the $+\Delta\phi$ s were always greater than that for the $-\Delta\phi$ s, and the data strongly suggested that the minimum for both was close to Ct 10.

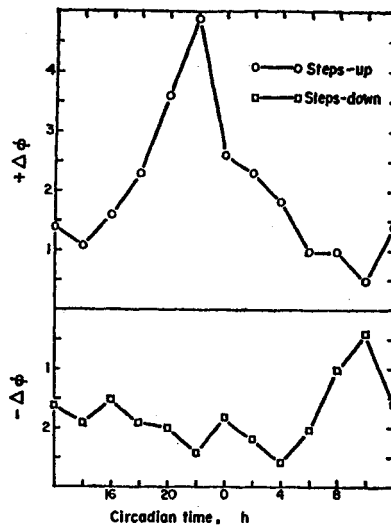


Fig. 3.41. Phase shift response curves for temperature steps up (20 - 28°C) and temperature steps down (28 - 20°C) in the *D. pseudoobscura* eclosion rhythm. Pupae were systematically exposed to temperature steps during the first 24 hours of DD free run (Ct 12 of day 0 to Ct 12 of day 1) after prior entrainment to LD 12:12 at either constant 20° or 28°C. $\Delta\phi$ values for temperature steps down are the average of $\Delta\phi$ s (medians of experimental minus that of free-run control) for days 6, 7 and 8; for temperature steps up, the $\Delta\phi$ values are the average of $\Delta\phi$ s for days 4 and 5. (From Zimmerman et al., 1968).

Just as the $\Delta\phi$ s for the 'on' and the 'off' signals in a light-cycle, as simulated by separate 15-minute light pulses in a skeleton photoperiod n hours apart, may be summed to calculate the net $\Delta\phi$ for a light pulse n hours long, so the $\Delta\phi$ s for temperature steps-up and steps-down may be summed to determine the net $\Delta\phi$ for temperature pulses. Thus the net $\Delta\phi$ for a high-temperature pulse (HTP), consisting of a 12-hour period at 28° in an otherwise 20° culture, was a sum of the advance and delay phase-shifts caused by the two steps. The net $\Delta\phi$ was, of course, a function of the circadian time at which the pulse was seen (Fig. 3.42), with phase advances occurring when the HTP was initiated at points between Ct 17 and Ct 05, and phase delays when the HTP was initiated between Ct 05 and Ct 17. Conversely, the phase-response curve for low-temperature pulses (LTP) showed phase delays between Ct 17 and Ct 05 and phase advances between Ct 05 and Ct 17. That separate $\Delta\phi$ s for the two steps may be used to determine the net $\Delta\phi$ for a pulse suggests that the phase-shifts are accomplished quite

rapidly (within a few hours). These results, therefore, show that temperature changes act as *Zeitgeber* in a manner comparable to changes from dark to light, or changes in light intensity.

Other investigators have examined the phase responses of *D. pseudoobscura* to temperature pulses, sometimes with apparently conflicting results. Chandrashekar (1974) studied the effects of 3-, 6- and 12-hour high (20-30-20°C) and low (20-10-20°C) temperature pulses, systematically scanning the whole circadian cycle. The results were generally in agreement with those of Zimmerman et al. (1968), although delays were always greater than advances. A 12-hour HTP caused the greatest $-\Delta\phi$ of about 5 hours. Plotting the response curves according to onsets, midpoints, or offsets of the pulses indicated that the midpoint treatment was the most 'meaningful' since it allowed better comparisons between pulses of different duration. It is also interesting to note that LTPs of 12 and 6 hours, starting at Ct 4 and Ct 10 respectively, caused arrhythmicity reminiscent of that induced by critical light signals falling on the pacemaker's singularity (see Chapter 3, E).

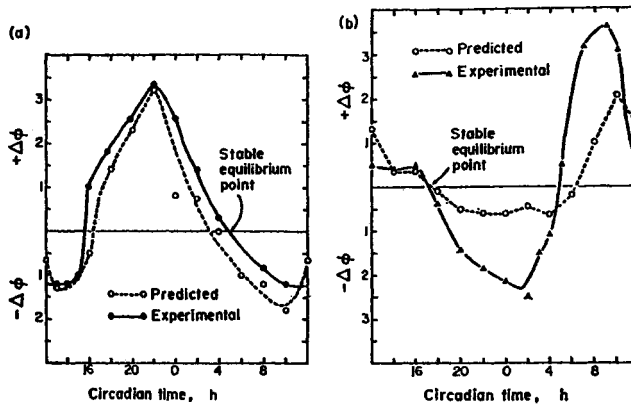


Fig. 3.42. Experimental and predicted $\Delta\phi$ curves for (a) single 12 hour high temperature pulses (HTPs)(20-28-20°C) and (b) single 12 hour low temperature pulses (LTPs)(28-20-28°C) in the *D. pseudoobscura* eclosion rhythm. Pupae were systematically exposed to temperature pulses during the first 24 hours of DD free run (Ct 12 of day 0 to Ct 12 of day 1) after prior entrainment to LD 12:12 at a constant 20° or 28°C. $\Delta\phi$ values for HTPs are the average of $\Delta\phi$ s for days 7 and 8; those for LTPs the $\Delta\phi$ s the average of days 4 and 5. Predicted $\Delta\phi$ values were derived from the response curves for temperature steps as described in Fig. 3.41. (After Zimmerman et al., 1968).

In the studies of Zimmerman et al. (1968) and Chandrashekar (1974) the phase-response curves produced in response to both high- and low-temperature pulses were of the 'weak' Type 1, delays and advances never greater than about 5 hours. Using the same species, however, Winfree (1972b) obtained 'strong' Type 0 resetting after 12 hour HTPs (20-28-20°C), peaks of eclosion occurring about 12 + modulo τ hours after the ends of the pulses, regardless of the phase at which they started. Even 3-hour pulses gave phase shifts of -3 to +5 hours.

Zimmerman et al. (1968) concluded that the temperature-induced phase shifts were accomplished quite rapidly, at least within a few hours. Maier (1973) has since examined the rapidity of these shifts using very short pulses of high temperature (4 minutes at 40°C), applied to otherwise 20° DD cultures. Unlike the longer pulses used by Zimmerman et al. (1968) and Chandrashekar (1974) these pulses gave only delays, with a maximum of about 4 hours at Ct

12 and a minimum of about 1 hour between Ct 0 and Ct 3. These delays were apparently achieved almost immediately: when the HTP was followed by a series of 5-minute light-pulses, the light-pulse PRC so produced was almost exactly delayed by the amount predicted from the temperature PRC (Fig. 3.43). An experiment was also performed in which flies were transferred from LL to DD to initiate a rhythm, exposed to a 4-minute temperature pulse 3 hours later (at Ct 15), and then probed with light pulses whose intensity was equivalent to Winfree's critical stimulus, S^* (see Chapter 3, E). If the temperature pulse at Ct 15 had delayed the oscillation by the amount expected (2.7 hours), the 'singularity', T^*S^* , ought to have been delayed by the same amount, from 6.8 hours to about 9.5 hours after the LL/DD transition. The results showed that the most arrhythmic cultures occurred when S^* fell between 10.6 and 10.8 hours after the transfer to darkness; although somewhat equivocal, this result does strengthen the conclusion that the temperature pulse had an almost *immediate* phase-delaying effect on the pacemaker.

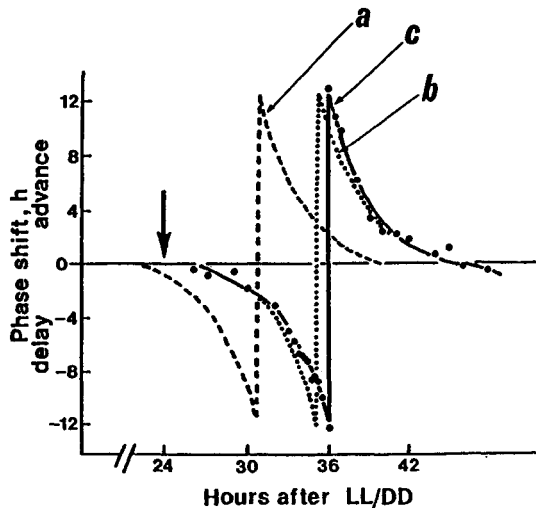


Fig. 3.43. *Drosophila pseudoobscura*: phase shifting effect of a high temperature pulse (HTP) (4 minutes at 40°C) on the circadian eclosion rhythm. Phase response curve is assayed after the temperature pulse by a succession of 5 minute light pulses. Vertical arrow – time of HTP; a – PRC for unperturbed cultures; b – predicted PRC following HTP; c – observed PRC following HTP. (From Maier, 1973).

The speed of this reset was investigated in a series of cultures in which the high-temperature pulses were placed closer and closer to T^* (6.8 hours) (Maier, 1973). All groups then received a light pulse of strength S^* 6.8 hours after LL/DD, the rationale behind this experiment being that if the temperature pulse shifted the oscillation, arrhythmicity would not occur with the singular combination of S^* (50 seconds) and T^* (6.8 hours after LL/DD). Results showed that all groups were rhythmic, even that in which the temperature pulse began 6.7 hours after the transfer to darkness (i.e. only 6 minutes before T^*). This suggested that temperature pulses – like those of light (Chapter 3, C.I) – have an almost immediate phase-shifting effect on the circadian pacemaker, rather than on some peripheral system (such as driven rhythm). Chandrashekar (1980) also concluded that temperature pulses phase shifted the circadian pacemaker. It should be borne in mind, however, that although these

interpretations are compelling, hitting the singularity in *D. pseudoobscura* is a difficult and delicate operation, and in many such experiments arrhythmicity remains low even with pulses of the necessary intensity, correctly timed (see Winfree, 1970a, Fig. 12).

Most of the evidence derived from studies of temperature pulses and cycles suggests that temperature changes reset the circadian pacemaker in a manner comparable to that by light. However, in the two-oscillator model conceived for *D. pseudoobscura* (Chapter 3, C.1) the pacemaker or A-oscillator is thought to be temperature-compensated and 'coupled' to the environmental light-cycle, whilst the driven element or B-oscillator was, in the original model (Pittendrigh and Bruce, 1957, 1959), thought to be temperature-sensitive and probably open to control by temperature cycles. Therefore, concurrent cycles of light and temperature might be expected to produce a 'conflict' in the steady state finally achieved. Using a sinusoidal temperature cycle (19–29°C) in conjunction with a light-cycle of LD 12:12, such an effect was observed (Pittendrigh, 1960). When the low point of the temperature cycle occurred close to dawn, as in the 'natural' situation, no disturbance of phase was observed. But as the phase angle between the two *Zeitgeber* was changed, the peak of pupal eclosion was delayed relative to the fixed light cycle until, at about 15 to 16 hours after dawn, a discrete 180° ψ -jump occurred. The results showed that out of the total conceivable 360° of phase, only 180° is realisable: there is a 180°, zone of 'forbidden' phase relations during which eclosion cannot occur.

The explanation for this abrupt change of phase may be found in the two-oscillator model for the clock underlying eclosion. For instance, when the system attempted to entrain to the two conflicting *Zeitgeber*, one part of the system (A) strictly followed the phase of the light-cycle, whereas another (B) was more influenced by the temperature cycle. There was clearly a limit to the phase-angle difference between these two subsystems: when that limit was reached the driven system was forced to leap back to its original phase close to dawn. A very similar result was obtained for the cockroach, *Leucophaea maderae* (Roberts, cited in Pittendrigh, 1960), and for the eclosion rhythm in *Pectinophora gossypiella* (Pittendrigh and Minis, 1971).

2. Is there a temperature-sensitive event between photoreceptor and clock?

Hamm et al. (1975) reported some original and ingenious experiments with *Drosophila pseudoobscura* in which flies were exposed to concurrent pulses of low temperature and light. Cultures of larvae maintained at 20°C were transferred from LL to DD to generate a rhythm of pupal eclosion, then subjected to 2 hours of reduced temperature (6°C) and a 100-second pulse of blue light (442 nm, 100 $\mu\text{W cm}^{-2}$) timed to occur 30 minutes after the start of the low-temperature pulse. The pairs of signals were then arranged, in different experimental groups, to occur at all circadian phases. The resulting phase-response curves (Fig. 3.44A) showed several differences from the 'standard' light-induced PRC without the accompanying cold pulse. After correction for the slight LTP-induced phase-shifts, the curve resembled the standard PRC more closely (Fig. 3.44B) in wave-form but not in its time-course. In short, the PRC was shifted in a manner which suggested that the light signals had 'arrived' at the oscillation about 1½ to 2 hours *later* than the phases at which they were apparently given. In other words, the results seemed to indicate a slowing down of the 'information transfer' between the photoreceptor and the clock. When the postulated 2-hour delay was taken into account, the experimental and 'standard' PRCs became identical (Fig. 3.44C).

In a second approach to the same phenomenon, using the 'singularity point' (Chapter 3E), it was argued that the critical light pulse S^* (10 seconds at 100 $\mu\text{W cm}^{-2}$ for this

population of *D. pseudoobscura*) would have to be given about 2 hours earlier than in the unchilled control if the interpretation given above was correct. Results showed precisely that: T^* for the chilled group was about 1½ to 2 hours ahead of that for the control. As an alternative to the conclusion that low temperature delays an event between the photoreceptor and the pacemaker, the authors considered the separate phase-shifting effects of the following events in the sequence in which they occurred; the step-down in temperature from 20° to 6°, the light-pulse occurring 30 minutes later, and the step-up from 6° to 20°. In the absence of their own data for temperature steps, phase shifts were estimated as being twice as effective, and half as effective, respectively, than those recorded by Zimmerman et al. (1968) (Chapter 3, G. 1) for the same species. None of the calculations based on these separate events were as close to the experimental data, however, and this alternative was rejected. No further work on this aspect of the eclosion rhythm has been reported.

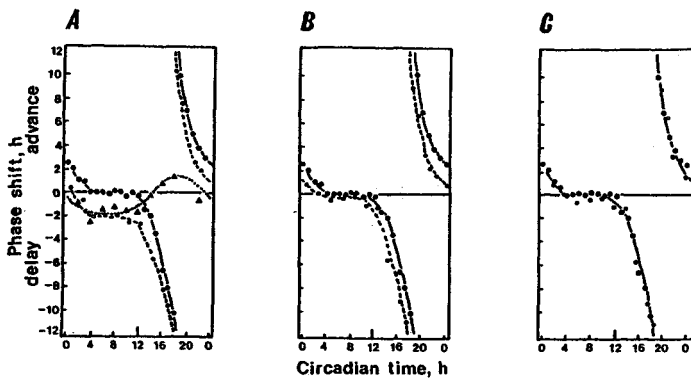


Fig. 3.44. *Drosophila pseudoobscura*, eclosion rhythm. Phase shifting action of light pulses (100 seconds) delivered during a low temperature pulse (2 hours at 6°C in otherwise 20°C). A – closed circles, PRC for 15 minute pulses (data from Fig. 3.17); open circles, PRC for 100 second pulses during the low temperature pulse; triangles, PRC for temperature pulse (2 hours at 6°C). B – same data for light pulse PRCs but with open circle curve (100 second light pulses delivered during the low temperature) corrected for the effects of low temperature alone. C – same data again, after correction for signal delaying action of the low temperature pulse (see text). (From Hamm et al., 1975).

3. Temperature cycles as Zeitgeber in the absence of light sensitivity

There are very few examples in which the light-cycle is not an important *Zeitgeber* for circadian rhythms: the leaf-cutter bee *Megachile rotundata* appears to be one such case (Tweedy and Stephen, 1970). This species diapauses as a 'pre-pupa' in a dense cocoon in a cell composed of several layers of leaf cuttings which, in turn, are enclosed in dark holes or cavities. Diapause is terminated by incubation at 17°C; the adult bees then emerge. In a light-cycle (LD 12:12) at constant temperature, eclosion is rhythmic but apparently free-runs across the light cycle with a period (τ) of 23.25 hours. The rhythm may be reset, however, by a cold pulse. In LL (108 lux) τ became 22.67 hours, but only in cultures in which rhythmicity was initiated by a cold shock; control cultures without such a temperature pulse were arrhythmic.

Another example concerns the rhythm of feeding and digestion in the log-infesting larvae of the beetle *Rhagium inquisitor* (Riba, 1976; Chapter 2, A.2), apparently entrained by

thermoperiod but not by light. Pupal eclosion in the tsetse fly *Glossina morsitans* may also be entrained by a temperature cycle but not by light (Zdarek and Denlinger, 1995).

C. SPECTRAL SENSITIVITY AND INTENSITY EFFECTS

Action spectra for light-induced phase-shifts of the *D. pseudoobscura* oscillation have been determined by Frank and Zimmerman (1969). Larval cultures of mixed developmental age were raised in LD 12:12 and then transferred to DD as pupae. Pulses consisting of 15 minutes of monochromatic light were then applied to the free-running oscillation at two circadian times. In one series the pulse was placed at Ct 17 where white light generates a phase-delay ($-\Delta\phi$); in a second series it was placed at Ct 20 to generate a phase-advance ($+\Delta\phi$). Control groups experienced either white light pulses at Ct 17 and 20, or were allowed to free-run unperturbed in DD. The $\Delta\phi$ s achieved with the monochromatic pulses were expressed as a percentage of the $\Delta\phi$ caused by white light at that point. The intensities of the monochromatic pulse were systematically altered until a steady-state (7 days after the signal) equalled 50 per cent of the white light control.

The results showed that the direction of the phase-shift (i.e. advance or delay) was not affected by wavelength, but the magnitude of the response increased with the intensity of the signal. The action spectra for both $+\Delta\phi$ and $-\Delta\phi$ were similar. The most effective wavelengths were between 420 and 480 nm (Fig. 3.45). Above 500 nm there was a sharp 'cut-off' and no response could be achieved with an intense non-monochromatic light spanning a broad spectrum range from 600 nm into the infrared. A more detailed action spectrum for phase delays (at Ct 17), showing essentially the same result, has been prepared by Klemm and Ninnemann (1976).

Although Frank and Zimmerman found no significant differences between delays and advances, with regard to the energy levels required to elicit a 50 per cent response, Chandrashekar and Engelmann (1973) have reported that Ct 19 (advances) was about 10 times 'more sensitive' than Ct 18 (delays) to pulses of blue light (442 nm).

Working with the initiation of the egg-hatch rhythm in *P. gossypiella*, Bruce and Minis (1969) found a very similar spectral sensitivity. In this study, populations of eggs were exposed to short light signals of monochromatic light after the midpoint of embryogenesis. The most effective wavelengths were between 390 and 480 nm; those above 520 nm were ineffective (Fig. 3.46).

Chandrashekar and Loher (1969b) exposed populations of *D. pseudoobscura* to white light-pulses of different intensities at Ct 15.5 ($-\Delta\phi$ of ~5 hours) and at Ct 21.5 ($+\Delta\phi$ of ~5 hours). They found that the magnitude of the $\Delta\phi$ s - both advance and delay - was independent of intensity above about 10 lux; the same phase-shifts were achieved, for instance, with 10, 3000 and 10,000 lux. Pulses between 10 lux and 1.0 lux caused $\Delta\phi$ s of the correct sign but of a smaller magnitude, but below 1.0 lux only delays ($-\Delta\phi$) were discernible. Moreover, when the transients occurring between the day of treatment and the final steady state (days 4 or 5) were examined, low light intensities (0.1, 0.3 and 1.0 lux) were found to cause temporary 'reversing transients' in a direction opposite to that finally achieved, or generated by higher intensities. These reversing transients were particularly clear in populations exposed to pulses at Ct 21.5 ($+\Delta\phi$ s). Their significance, however, remains obscure.

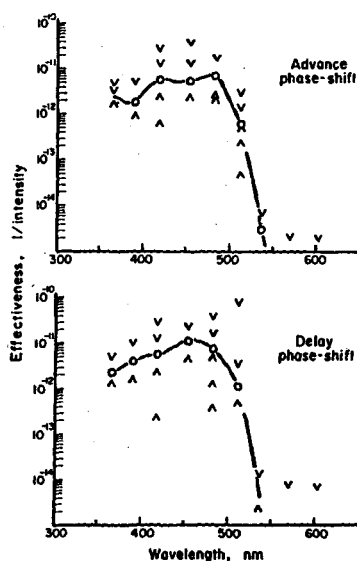


Fig. 3.45 (left). Action spectrum for 50 per cent phase shifts of the *D. pseudoobscura* eclosion rhythm induced by 15 minute light signals: Upper panel – advance phase shifts; Lower panel – delay phase shifts. (From Frank and Zimmerman, 1969)(Copyright 1969 by the American Association for the Advancement of Science).

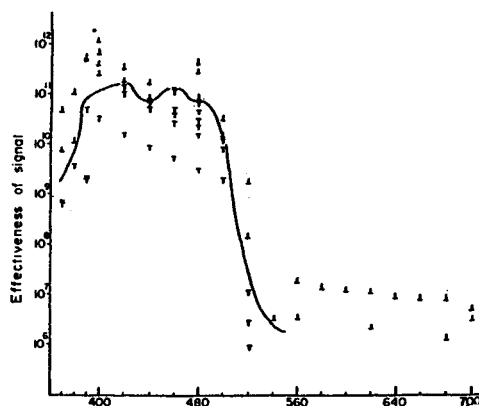


Fig. 3.46 (right). Action spectrum for the initiation of the rhythm of egg hatching in *Pectenophora gossypiella*. (From Bruce and Minis, 1969)(Copyright 1969 by the American Association for the Advancement of Science).

Engelmann (1969) similarly examined the phase-shifting effects of blue light (440 nm) falling at Ct 16 and Ct 20, varying the duration of the pulses from 1/100 second to 15 minutes, and the energy from 0.5 to 5000 ergs $\text{cm}^{-2} \text{sec}^{-1}$. He found no significant phase-shifting effects at either phase with pulses below 10^2 ergs cm^{-2} , but above this level pulses falling at Ct 16 caused phase delays and pulses at Ct 20 phase advances, with the magnitude of $\Delta\phi$ being a function of signal energy. Above about 10^4 ergs cm^{-2} the phase-shifting response was apparently saturated.

The peak of sensitivity at 420 to 500 nm might suggest that a carotenoid pigment was involved in photoreception. However, Zimmerman and Goldsmith (1971) raised larvae of *D. melanogaster* on aseptic diets with and without β -carotene, and tested the adults for the photosensitivity of the compound eyes and the photosensitivity of the circadian rhythm controlling adult eclosion. Visual sensitivity was assayed by measuring the height in millivolts of the sustained corneal negative wave elicited in the dark-adapted eye by monochromatic light (454 nm) in pulses of approximately 1 second. The photosensitivity of the circadian rhythm was assayed by measuring the $-\Delta\phi$ generated by a 15-minute light signal of monochromatic light (454 or 458 nm) applied to a population of pupae at Ct 15. Since carotenoids are only synthesised by plants, and carotenoid-deficiency in insect diets results in a loss of visual sensitivity, a similar impairment of the circadian oscillation might be expected if a carotenoid-derived chromophore was also involved in the latter system. The results, however, showed that although photosensitivity of the compound eyes in the carotenoid-depleted flies was about 3 log units lower, the responses of the circadian system in both groups were identical. Therefore, unless sufficient carotenoid was passed through the egg and used preferentially for the circadian rhythm chromophore, these results suggested that carotenoids were not involved in

the phase-shifting of the *D. melanogaster* oscillator. This conclusion was supported by the fact that the action spectrum for phase-shifts (Frank and Zimmerman, 1969) was quite different to that for the compound eyes, and also quite different from the absorption spectrum for common carotenoids. The identity of possible photoreceptor molecules will be further examined in Chapter 4.

D. SOME GENETIC ASPECTS OF POPULATION RHYTHMS

1. Latitudinal clines in clock parameters

Using the pupal eclosion rhythm in *Drosophila* spp. several important studies (Lankinen, 1986a, 1993a; Pittendrigh and Takamura, 1989) have investigated the natural genetic variations occurring along latitudinal (south-north) clines.

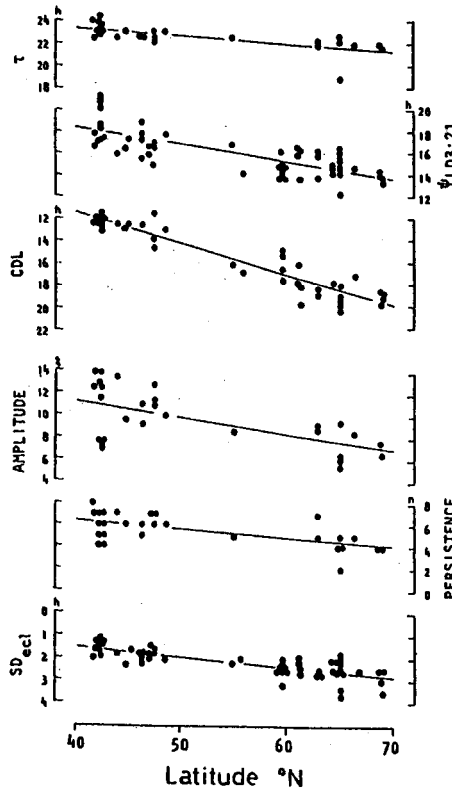


Fig. 3.47. *Drosophila littoralis*. Latitudinal clines of the period of the free running rhythm (τ), phase of the entrained eclosion rhythm at LD 3:21 ($\Psi_{LD\ 3:21}$), critical day length (CDL), amplitude and persistence of the free running rhythm of eclosion, and amplitude of the entrained eclosion rhythm at LD 3:21 (SD_{ecl}). (From Lankinen, 1986a).

Lankinen (1986a) studied 57 strains of *D. littoralis* collected from an immense latitudinal range from Batumi on the Black Sea coast (41° 35'N) to northern Finland (69°N),

and examined the various populations of flies for characters such as phase relationship of the peak of eclosion to light (ψ_{RL}); free-running period in darkness (τ_{DD}); 'amplitude' of the eclosion peaks; and PRC shape. Despite some local variation, ψ_{RL} in LD 3:21 showed that more northerly strains emerged *earlier* in relation to the 3 hour light pulse than strains to the south (Fig. 3.47). The free-running period, initiated by a transfer of cultures from LL to DD, was *longer* in the south than in the north (range, 18.8 to 24.3 hours), a difference which, because of the relationship between ψ and τ referred to earlier (see this Chapter, section C 2), probably contributed to earlier eclosion in the more northerly populations. The 'amplitude' of the eclosion peaks (i.e. the number of flies using the gate) was higher in the south, and the phase response curve (for 1 hour pulses of white light at 500 lux), was a 'weak' Type 1 for a strain isolated in Zurich (47°15'N), whereas in a strain isolated further south (Batumi, 41°35') it approached a stronger, Type 0.

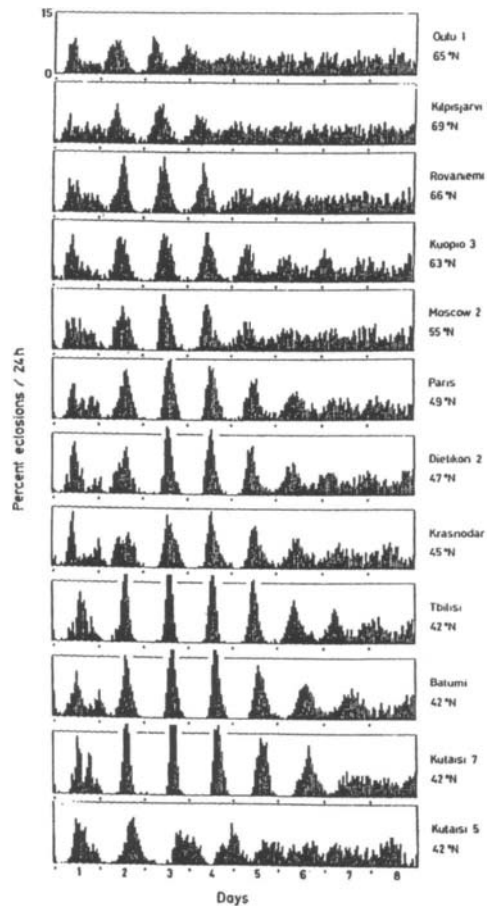


Fig. 3.48. *Drosophila littoralis*. Free running rhythms of eclosion from different latitudes showing overt damping in northern strains. The rhythms were initiated by a transfer from LL to DD (19°C) at the beginning of day 1. (From Lankinen, 1986a).

Another important difference was that all strains showed evidence of rhythm damping, most markedly to the north where some strains became almost totally arrhythmic after about 3 cycles in DD (Fig. 3.48). Lankinen attributed this arrhythmicity to an uncoupling between pacemaker (A) and slave (B) (see this Chapter, section C 1), leading to 'secondary arrhythmicity' (Zimmerman, 1969) in each pupa, and to random phases in the population. These north-south differences in circadian rhythm parameters, together with similar data for critical day length, will be discussed further in Chapter 11 with reference to the photoperiodic regulation of diapause.

In a later study of *D. subobscura*, Lankinen (1993a) investigated 12 strains from two geographical areas: Scandinavia (56 to 63°N) and the Canary Islands (28°N). Although not a continuous north-south series, the data resembled those for *D. littoralis*: in northern populations, ψ_{RL} was earlier, τ_{DD} was shorter, and 'amplitude' lower than in the south. Early eclosion was also correlated with a short value of τ ($r = 0.76$).

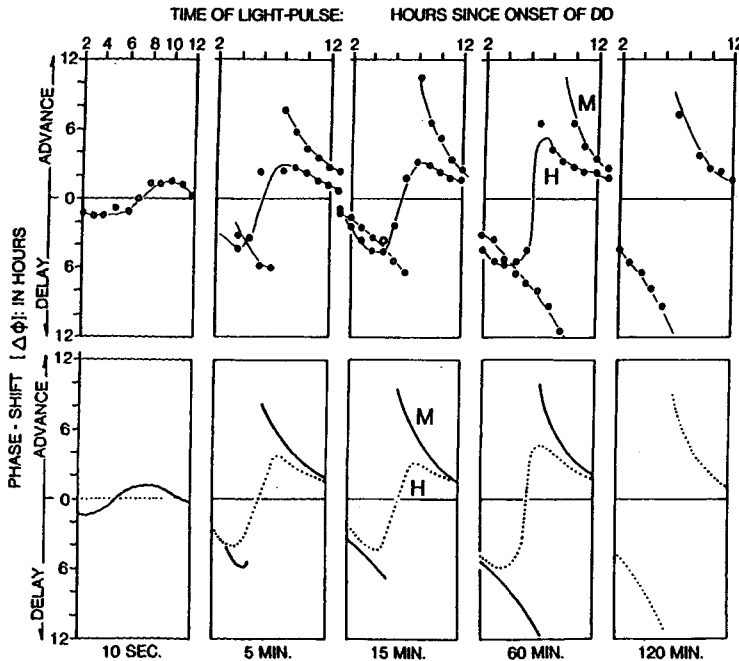


Fig. 3.49. *Drosophila auraria*, eclosion rhythms. Upper panels – phase response curves (PRCs) for a strain from Hokkaido (43°N) (H; dots) and a strain from Miyake (34°N) (M; circles). Each panel gives curves for a particular pulse duration. All 'intensities' are 50 lux. Lower panels – Hokkaido PRCs (stippled) reproduced from upper panels; Miyake PRCs (solid) shifted 2 hours to the left. (From Pittendrigh and Takamura, 1989).

Pittendrigh and Takamura (1989) studied latitudinal variation of eclosion rhythm phenotypes in populations of *D. auraria* isolated from Japan, over a range from Miyake (34.2°N) to Hokkaido (42.9°N); the range of latitudes was therefore smaller than that in Lankinen's (1986a) study of *D. littoralis*, and it extended further south. The *D. auraria* study

revealed some similarities, and also some differences, to that for *D. littoralis*. For ψ_{RL} in a daily photoperiod of LD 14:10, for example, the more northerly strain phase-led that from the south, as expected, but in LD 1:23 Hokkaido phase-lagged Miyake. A second difference was that τ_{DD} was longer to the north, rather than shorter. In agreement with *D. littoralis*, however, the PRC (for 15 minute pulses at 50 lux) for Hokkaido was of lower amplitude ('weak', Type 1) than that for Miyake ('strong', Type 0). Only when the pulse duration was increased from 15 to 120 minutes was the Hokkaido PRC comparable to that for the more southerly strain (Fig. 3.49). Pittendrigh and Takamura likened this difference to a lowered "subjective light intensity" in flies from the north. They considered that this latitudinal cline in PRC shape had a functional significance in conserving the amplitude of the pacemaker's signal to the rest of the system it times. The reduction in subjective light intensity seen to the north would thus serve to compensate for the overall increase in photoperiod occurring in the north during the breeding season.

As with Lankinen's study of latitudinal variation in *D. littoralis*, Pittendrigh and Takamura (1989) extended their analysis to include observations on the photoperiodic induction of diapause, and the possible relationship of day length measurement to the circadian system: we will return to this topic in Chapter 11.

2. Selection for phase angle: 'early' and 'late'

The non-steady-state (transient) cycles which follow perturbation with light-pulses, and the 'conflict' generated when the phase-angle between opposing light and temperature cycles is varied, suggest the participation of at least two oscillators ('pacemaker' and 'slave') in the eclosion clock of *D. pseudoobscura* (see section C 1). The existence and full significance of these subsystems have been made clear by certain types of genetic experiment. The first of these involved the differential selection of flies emerging either 'early' or 'late' in the daily eclosion gate.

Pittendrigh (1967) systematically bred from parents that emerged either very early or very late in the daily distribution of emergence activity in populations kept at LD 12:12. After fifty generations of such selection the difference in eclosion time between the two artificially selected strains was about 4 hours (see Fig. 6.13). This difference in phase angle was maintained under all photoperiods. However, when the phase response curves for the two strains were measured they were found to be identical. Therefore, selection had clearly altered the phase angle between the slave and the pacemaker, but not between the pacemaker and the light. This demonstrated the reality of the two-oscillator system proposed in 1957 by Pittendrigh and Bruce, and made the distinction between the light-sensitive driving *oscillation* (A) and the driven *rhythm* (B) abundantly clear. These data will be considered further in Chapter 6.

This important observation was repeated for the pink bollworm moth *Pectinophora gossypiella* (Pittendrigh and Minis, 1971). In this species selection resulted in an 'early' strain emerging from its pupae about 5 hours before 'late'. Selection for eclosion time has also been achieved for *D. melanogaster* (Clayton and Paietta, 1972) and for the blow fly *Calliphora vicina* (Zinovjeva and Polyakova, 1987). These results lead to a concept of an adaptively adjustable coupling of driven systems to the common driving oscillation, and to the concept of the 'circadian system' which will be examined further in Chapter 6.

3. Heavily damped rhythms and arrhythmicity

Pupal eclosion rhythms, such as those described in this chapter for *D. pseudoobscura* (Pittendrigh, 1954) and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1978a), are normally robust and persistent. Some insects, however, particularly these from more northerly latitudes, may show pronounced damping of their rhythms, often leading to an overt arrhythmicity. Such arrhythmicity appears to be different from that brought about by continuous light of high intensity (see section D); it may be akin to Zimmerman's (1969) 'primary arrhythmicity' (see section E).

Working with the pupal eclosion rhythm of the fruit fly *Drosophila littoralis*, Lankinen (1986a) found that rhythm damping, often within 2 to 7 cycles in DD, was of widespread occurrence, but particularly so amongst strains isolated from more northerly latitudes (Fig. 3.48). A natural damping was also recorded in a northern strain of the midge *Clunio marinus* (Pflüger and Neuman, 1971). In the drosophilid *Chymomyza costata* (Lankinen and Riihimaa, 1992, 1997) eclosion rhythmicity was always weak, even in a diel photoperiod, and damped out within 2 to 3 cycles in constant conditions. Smith (1985) recorded a naturally occurring arrhythmic strain of the blow fly, *Lucilia cuprina*.

Lankinen (1993) described a particularly interesting mutant of *D. subobscura*, named *linne*. This mutation was autosomal and recessive; flies homozygous for this gene showed a weak circadian rhythm of eclosion that was not entrainable by a light cycle. However, it could be entrained using a temperature cycle of only 4°C amplitude, and this rhythm then persisted in both LL and DD. The mutation is thought to disrupt the coupling between pacemaker and light, rather than the pacemaker itself.

4. Isolation of 'clock' mutants

The treatment of *Drosophila melanogaster* with the chemical mutagen, ethyl methane sulphonate (EMS) by Konopka and Benzer (1971) resulted in the isolation of three *period* or 'clock' mutants. One of these was arrhythmic (*per*⁰); the other two were rhythmic but had free-running periods (τ) of about 19 hours (*per*^S) and about 28 hours (*per*^L), respectively. These altered periods were evident in both eclosion and in the rhythm of adult locomotor activity. Since the latter was recorded using individual insects, the arrhythmicity of *per*⁰ was not due to a desynchronisation of individuals in the population, although it might have been due to a desynchronisation of separate circadian subsystems within the individual. This very influential paper by Konopka and Benzer has led to a very large literature on the genetics and molecular biology of the feedback loops thought to provide the 'clockwork' for circadian rhythmicity in *D. melanogaster* and other insects. Developments in this field will be reviewed in Chapter 4.

ANNOTATED SUMMARY

1. 'Once-in-a-lifetime' developmental events, such as egg hatching, moulting from one larval instar to another, pupation, or pupal eclosion, are frequently controlled by an endogenous circadian oscillator (pacemaker) which only manifests itself in a mixed-age population of insects.
2. These rhythms are generally 'damped out' by protracted periods of light, but may be generated or initiated by a single step-wise transfer from light to darkness, a single

- exposure to a light or temperature pulse, or by the transfer from successive light/dark cycles into continuous darkness. The free-running rhythm so revealed exhibits its natural or endogenous period (τ) which is frequently close to 24 hours.
3. The free-running circadian period τ is temperature-compensated, with a Q_{10} close to 1. It is also homeostatically buffered against many other changes in the physiological milieu, although heavy water (D_2O) and agents such as lithium may cause dose-dependent lengthening of τ .
 4. In 'population rhythms' the circadian oscillation in each insect dictates 'allowed zones' or gates through which eclosion, egg hatching, or hormone release may occur. In a mixed-age culture this mechanism partitions the population into daily activity peaks. In continuous darkness (DD) the free-running oscillation produces successive gates at intervals of modulo τ .
 5. A free-running oscillation perturbed by light (or temperature) pulses shows steady-state phase advances ($+\Delta\phi$) or phase delays ($-\Delta\phi$) depending on the phase of the oscillation so perturbed. Phase delays are generated by pulses falling in the early 'subjective night'; phase advances by pulses in the *late* 'subjective night'. A plot of such phase changes against the circadian time of the perturbation gives a phase response curve (PRC) for the oscillation.
 6. After a single unrepeatable light perturbation the overt rhythm generally undergoes several transient cycles before reaching its new steady state. The number of such transients is usually greater in the case of phase advances. Pittendrigh's model to account for such phenomena envisages an A-oscillator (the circadian 'pacemaker') which is immediately (i.e. within minutes) reset by the light pulse, and a driven element, the B-oscillator, which is coupled to A on the one hand, and to the physiological processes controlling eclosion, hatching, etc., on the other.
 7. Whereas the phase relationship between the pacemaker and the light cycle (ψ_{PL}) is determined by the PRC, the phase relationship between the observed eclosion peak and the pacemaker (ψ_{EP}) is flexible, eclosion occurring later relative to dawn as the temperature is lowered. A flexible ψ_{EP} is also evident in different photoperiods or in light cycles with a different period. Genetic selection for 'early' and 'late' eclosion within the daily gate also indicates the flexibility between pacemaker and slave.
 8. 'Weak' (i.e. short or less intense) light pulses may give rise to low-'amplitude' PRCs of Winfree's Type 1. 'Strong' (long or bright) pulses may give rise to Type 0 PRCs. Type 1 curves may give way to Type 0 curves quite abruptly: in *Drosophila pseudoobscura* the change occurs with about 50 seconds of blue light at an intensity of about $10 \mu W cm^{-2}$. Pulses whose 'strengths' are intermediate between Type 1 and 0, and applied at a critical phase of the oscillation, may place the oscillation on its 'singularity', where overt rhythmicity is not observed. At this point the circadian pacemaker appears to be in a 'phase-less' state (i.e. the clock has been 'stopped'), equivalent to the arrhythmicity observed in cultures raised in DD.
 9. The free-running oscillator can be entrained by a variety of light-cycles, including repeated pulses defining environmental cycles of different period (T), or two or more pulses per cycle forming 'skeleton' photoperiods. In each case the endogenous periodicity (τ) is adjusted to that of the environmental driving cycle (T) by discrete and instantaneous phase-shifts caused by the light transitions. The phase-shifts generated, and the process of entrainment, can be calculated from the phase-response curves.

10. Both the light-on signal and the light-off signal of a pulse cause phase shifts, but the former is 'dominant' in the late subjective night, whereas the latter is 'dominant' in the early subjective night.
11. Extended period of light of sufficient intensity may cause 'damping out' to eventual arrhythmicity. Transfer to darkness then re-initiates the rhythm of eclosion with the phase of the pacemaker oscillation extrapolating back to Circadian time (Ct) 12 at the LL/DD transition. The apparent phase resetting of the oscillation at this point, however, also occurs after extended periods of light at an intensity too low to eliminate the overt rhythm. It is concluded that LL, except at the highest intensities, fails to damp out the motion of the pacemaker, only the slave rhythm and the overt event (eclosion) that it times.
12. Latitudinal (south-north) clines in eclosion rhythm parameters are known for several species in which more northerly strains have a shorter period (τ), an earlier eclosion in relation to the daily light cycle, a lower 'amplitude', and a weaker (more Type 1) PRC, than strains to the south. The reduced 'subjective light intensity' evident in more northerly strains might be an adaptation to compensate for the greatly extended daylight hours of the northern summer.
13. Circadian oscillations may also be entrained and phase-shifted by temperature pulses and cycles, temperature steps-up generally simulating 'light-on' and steps-down simulating 'light-off'.
14. In *D. pseudoobscura* phase-shifts of the A-oscillator may be generated by light intensities down to less than 1 lux. Action spectra for both advance and delay phase-shifts are similar and show maximum sensitivity between 420 and 480 nm, with a sharp 'cut-off' above 500 nm.

CHAPTER 4

CIRCADIAN RHYTHMS: GENES AND THE FEEDBACK LOOP

The Darwinian Demon has certainly had plenty of physiologic oscillations to work with, because his commonest device in installing regulators – from the control of heartbeat to that of protein synthesis – is negative feedback. And one of the innate tendencies of such feedback systems is to oscillate.

C. S. Pittendrigh, The Harvey Lectures 1961

CONTENTS

Introduction	103
A. <i>The Concept of Negative Feedback Loops</i>	104
B. <i>Some Important Genes Involved in Circadian Rhythms</i>	104
1. Presumed ‘central’ or ‘clock genes’	104
2. Genes on the input pathway	107
3. Genes on the output pathway	108
C. <i>Spatial and Temporal Clock Gene Expression</i>	109
D. <i>The Current Molecular Model for Circadian Rhythmicity</i>	110
E. <i>Possible Explanations of Canonical Circadian Properties</i>	110
1. Circadian period	111
2. Temperature compensation	111
3. Phase resetting by light	112
Annotated Summary	112

INTRODUCTION

THE paper by Konopka and Benzer (1971) - introduced at the end of Chapter 3 - demonstrated for the first time *in any organism* that complicated rhythmic behaviours could be regulated or affected by the action of a single gene – the *period* gene. Three alleles of *per* were originally isolated following chemical mutagenesis of *Drosophila melanogaster*: a short period gene (*per^S*) with a free-running period of about 19 hours in darkness (τ_{DD}), a longer-than-normal *per^L* with τ_{DD} of about 29 hours, and a third, apparently arrhythmic, *per^O*. In every way this paper may be regarded as a truly ‘seminal’ paper in the sense that it was entirely original, and subsequently gave rise to a voluminous literature generating enormous advances in our knowledge of the genetical and molecular aspects of circadian rhythmicity. The full range of this very large literature is outside the scope of this book whose aims are to present the properties and functions of insect circadian systems with a view to underpinning our knowledge and appreciation of overt behavioural rhythms (Chapters 2 and 3; Chapters 5

to 8) and the probable role of the circadian system in seasonal photoperiodism (Chapter 9 to 13). This large literature, however, has been discussed repeatedly in a number of important reviews (e.g. Hall, 1998; Dunlap, 1999; Rosato and Kyriacou, 2001)) and in at least one book dedicated to the subject (Sehgal, due in 2003). Readers wishing to follow up this extensive literature should consult these sources and the references contained therein. This chapter therefore is presented as a *linking* chapter, painting the molecular aspects of the circadian rhythm story in broad brush strokes, leaving most of the detailed molecular genetics to be found in the reviews just cited.

A. THE CONCEPT OF NEGATIVE FEEDBACK LOOPS

The idea that a largely negative feedback loop regulated circadian rhythms in plants and animals has had a long history (see also Chapter 7), as exemplified by the quotation that heads this chapter. Clearly, early writers such as C.S. Pittendrigh envisaged protein synthesis to be a crucial element in such a loop, but it was not until *period* and the other genes involved in the circadian clock were discovered and characterised was this concept to crystallise into more specific molecular feedback models involving gene transcription and translation. In such models the essential hypothesis was that the product(s) of participating gene(s) fed back to negatively regulate their own transcription. How this occurred, the identity of the genes involved, and how such a system could explain the defining circadian properties - near-24 hour periodicity, persistence under constant conditions, temperature compensation of the circadian period, and the entrainment of these rhythms to environmental signals of light and temperature - had then to be elucidated.

B. SOME IMPORTANT GENES INVOLVED IN CIRCADIAN RHYTHMS

1. Presumed central or 'clock' genes

At least five genes are thought to be involved in a transcription-translation feedback loop giving rise to the so-called 'central' oscillator; these are: *period*, *timeless*, *Clock*, *cycle*, *doubletime*. These genes, together with an account of their main phenotypes, will be described here before attempting to assemble their actions into a meaningful circadian oscillator (section D). These 'central' genes, with some others intimately involved with circadian rhythmicity, are listed in Table 4.1. Table 4.2 shows some of these genes' most important mutant alleles.

Apart from the three original alleles of *period* (Konopka and Benzer, 1971) other mutant alleles have now been described, the most important of which are further arrhythmic or apparently arrhythmic mutants (now called *per*^{O1} to *per*^{O4}) and an ultrashort *per*^T with a free-running period of about 16 hours. These variations in *per* are expressed in both locomotion and eclosion. That *per* is part of a negative feedback loop was strongly indicated by Hardin et al. (1990) who showed that *per* mRNA abundance cycled with a circadian frequency - about 19 hours in *per*^S and about 29 hours in *per*^L; mRNA was expressed randomly in *per*^O. In a light-dark cycle, RNA levels were highest at the end of the day (Zt 13 to 16) or early in the night. PER protein levels also cycled, but with a maximum about 6 hours later than the corresponding mRNA, strongly suggesting feedback of PER protein on transcription.

TABLE 4.1. Circadian 'clock' genes in *Drosophila melanogaster*. Only the most important are listed, i.e. those playing a significant role in the central feedback loop for adult locomotor activity and pupal eclosion, or on their input and output pathways. L – locomotor activity; E – eclosion.

Gene	Clock role	Key references
<i>period</i>	negative regulator	Konopka & Benzer, 1971
<i>timeless</i>	negative regulator, and light sensitive	Sehgal et al., 1994
<i>Clock</i> <i>cycle</i>	positive regulators	Allada et al., 1998 Rutila et al., 1998
<i>doubletime</i>	phosphorylates PER protein	Price et al., 1998
<i>vrrille</i>	blocks <i>per</i> and <i>tim</i> expression	Blau and Young, 1999
<i>cryptochrome</i>	photoreceptor; input pathway	Stanewsky et al., 1998
<i>lark</i>	output pathway; eclosion	Newby & Jackson, 1993
<i>pigment dispersing factor</i>	output pathway; locomotion	Renn et al., 1999
<i>takeout</i>	output pathway; feeding?	Sarov-Blat et al., 2000

Sequencing of the *per* gene revealed no conventional DNA binding domain in the PER protein which clearly would be required for negative regulation. However, a characteristic PAS domain (named for the proteins it was first found in - namely PER, ARNT and SINGLE-MINDED) was apparent. Therefore, although PER lacked a conventional DNA binding domain, the possession of the PAS sequence suggested an action through protein-protein associations, and the hunt for additional 'clock' genes and their products was on.

Until the early 1990s, *period* was the only recognised 'clock' gene in *D. melanogaster*. In 1994, however, *timeless* (*tim*) was discovered (Sehgal et al., 1994; Sehgal et al., 1995) whose null mutation *tim*⁰, like *per*⁰, produced apparently total arrhythmia. Long (*tim*^L), short (*tim*^S) and some other variants were also soon uncovered. *tim* mRNA abundance oscillated in tandem with that of *per* with a maximum at the end of the day or beginning of the night. PER and TIM proteins then reached a maximum in the middle of the night, at Zt 18 to 24. PER levels remained high until early morning, but TIM levels declined somewhat earlier. In both *per*⁰ and *tim*⁰ flies, RNA and protein cycling was abolished. TIM protein, like PER, contained a PAS domain allowing the two proteins to mutually bind in the cytoplasm to form a heterodimer. It was postulated that this protein complex was then able to translocate back into the nucleus to complete a negative feedback loop (see below).

TABLE 4.2. Some mutant phenotypes of important ‘clock’ genes. L – locomotion; E – eclosion.

Gene	Mutant allele	Mutant phenotype	Key references
<i>period</i>	<i>per^S</i>	$\tau \sim 19$ h; L and E	Konopka & Benzer, 1971
	<i>per^{L1}</i>	$\tau \sim 29$ h; L and E	ditto
	<i>per^{L2}</i>	ditto	Konopka, 1987
	<i>per⁰¹⁻⁰⁴</i>	arrhythmic; L and E	Konopka & Benzer, 1971 Hamblen-Coyle et al., 1989
	<i>per^T</i> <i>per^{clk}</i>	$\tau \sim 16$ h; L and E $\tau \sim 22.5$ h; L and E	Konopka et al., 1994 Dushay et al., 1990
<i>timeless</i>	<i>tim⁰</i>	arrhythmic	Sehgal et al., 1994
	<i>tim^S</i>	short period	
	<i>tim^L</i>	long period	
<i>Clock</i>	<i>Clk^{L-7}</i>	arrhythmic L	Allada et al., 1998
<i>cycle</i>	<i>cyc⁰¹⁻⁰²</i>	arrhythmic	Rutila et al., 1998
<i>doubletime</i>	<i>dbr^S</i>	short period	Price et al., 1998
	<i>dbt^L</i>	long period	
<i>cryptochrome</i>	<i>cry^b</i>	reduced light sensitivity	Stanewsky et al., 1998
<i>pigment dispersing factor</i>	<i>pdf⁰¹</i>	arrhythmic or abnormal L and E	Renn et al., 1999

Once again, however, the PER-TIM dimer lacks a conventional DNA-binding domain. The complex, however, seems to achieve transcriptional regulation by further associations with positive transcription factors. These factors are encoded by *Clock* (*Clk*) (Allada et al., 1998) and *cycle* (*cyc*) (Rutila et al., 1998). CLK and CYC proteins share with PER a protein-protein interaction or PAS domain but, unlike PER, also contain a basic helix-loop-helix (bHLH) region allowing binding to DNA at a short sequence called an E-box in the promotor. The E-box appears to be a key circadian regulatory element (Kyriacou and Rosato, 2000) allowing transcriptional repression of *per* and the closure of the circadian loop.

Other genes involved as elements within the proposed loop include *doubletime* (*dbt*) and *vri* (*vri*). The first of these, *doubletime* (Price et al., 1998), which encodes a casein kinase 1 ϵ , is not in itself rhythmically expressed but is a clock component in that two mutant alleles, *dbt^S* and *dbt^L* shorten and lengthen circadian periods by accelerating or delaying

phosphorylation of PER, respectively. Delayed phosphorylation might increase PER stability so that it persists longer, whereas accelerated phosphorylation might hasten protein turnover and thus shorten period. The last probable 'clock' gene is *vrille* (Blau and Young, 1999) which is expressed rhythmically in phase with *per* and *tim* and whose over-expression alters behavioural phenotypes consistent with it acting as a block to *per* and *tim* expression.

Recent genome-wide analyses of gene expression in *D. melanogaster* using microarrays (McDonald and Rosbash, 2001; Claridge-Chang et al., 2001; Ueda et al., 2002) have revealed a large number (several hundred) oscillating genes. These were found to include known, *bona fide*, 'clock' genes (see above) as well as a host of others involved in many biochemical and physiological processes, and some with an unknown function. This suggests that circadian rhythmicity is a widespread if not all-pervading physiological phenomenon. Many of these genes were located in gene clusters apparently subject to transcriptional co-regulation and control by *Clock* (*Clk*). Some of these cycling genes, however, might be coupled (as entrained slaves?) downstream of the central 'clock' (the pacemaker), but others could merely be passively *driven* elements. Still others could conceivably be 'clock' genes in their own right. Furthermore, since screening was based on robust and persistent gene expression, cycling genes giving rise to less persistent, damping oscillations may well have been overlooked. The idea that the insect genome might include circadian feedback systems independent of the best-known *per-tim* loop (see below) may have some validity, as suggested, for example, by apparently *per*-independent rhythmicity in the locomotor activity of *per*⁰ flies (Helfrich-Förster, 2001; Chapter 16). In short, we should not be surprised if the circadian system is even more complex than we currently realise. Most of the account given is based on the locomotor activity rhythm of *D. melanogaster* (Chapter 2) and this species' pupal eclosion rhythm (Chapter 3); both of these 'clocks' are brain-centred (see Chapter 8). Many other physiological systems in diverse tissues, however, are also rhythmic (see Chapters 5 and 6), adding to the complexity of the circadian system.

2. Genes on the input pathway

The traditional view of the input pathway is the route by which entraining signals from the environment (light, temperature and some 'non-photic' *Zeitgeber*) reach the 'clock' to adjust its period and phase to match the 'day outside' (see Chapters 2 and 3). Most of what we know about this route concerns light.

It is known that the fruit fly, *D. melanogaster*, uses multiple photoreceptive pathways for entrainment (see Chapter 8; Foster and Helfrich-Förster, 2001). These include both ocular (compound eyes) and extra-ocular photoreceptors (Hofbauer-Buchner eyelets; 'direct' brain photoreception); there is no compelling evidence that the simple eyes or ocelli are involved. Unlike some hemimetabola (cockroaches, for example) the compound eyes are not the dominant photoreceptors; neither may they be regarded as essential because eyeless mutants (*sine oculis*, *so*) readily entrain to the external light cycle. The so-called Hofbauer-Buchner (H.-B.) eyelets (Hofbauer and Buchner, 1988) are also present in these eyeless flies. They contain four photoreceptive, rhabdomere-like cells that may be immunolabelled by antibodies raised against rhodopsin, arrestin (a protein in the phototransduction cascade) and the 'clock' protein PER. The H.-B. eyelets also project to the central part of the insects brain which contains putative circadian pacemaker cells (the lateral neurons; Chapter 8) important for the regulation of locomotor rhythmicity. Light signals are also thought to reach the brain directly, probably straight to the lateral neurons.

The light entrainment pathway in *D. melanogaster* thus comprises at least three routes, one ocular and two extra-ocular. Having said that, there is much complexity in the light entrainment process. Almost all experimental studies have used simple ‘on-off’ pulses of white light against a background of darkness. In the natural environment, however, light at dawn and dusk – when entrainment takes place – occurs not only with gradual and directional changes in intensity, but also in quality (wavelength) and even in the position of the sun (Foster and Helfrich-Förster, 2001). Circadian photoreceptors must therefore be able to extract time-of-day information from complex twilight signals, reducing the incoming ‘noise’ to provide the insect with a coherent signal. This environmental complexity is discussed further in Chapter 16.

Carotenoids are probably involved as photoreceptive pigments in the compound eyes and H.-B. eyelets, but how does light reach the ‘central’ oscillator by the ‘direct’ route to the brain? In *Drosophila*, TIM protein responds to light, becoming degraded within 30 to 90 minutes of light exposure (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996); this suggests a mechanism by which light signals could alter the feedback loop directly. TIM protein possesses an action spectrum that resembles that for behavioural entrainment, but also interacts with another circadian photoreceptor – cryptochrome – in a light-dependent manner.

Cryptochrome (CRY) acts as a photoreceptor in *D. melanogaster* (Stanewsky et al., 1998). It is a blue-light absorbing protein expressed in the lateral neurons thought to be the location of the pacemaker for locomotor rhythmicity (see Chapter 8). *cry^b* mutant flies are unable to entrain to short light pulses and fail to become arrhythmic in constant light. Determination of the role of cryptochrome, however, is complicated in flies with normal compound eyes and H.-B. eyelets, because the photoreceptive pathways provided by these structures allow the flies to entrain in an almost normal fashion. In flies carrying *cry^b* and a mutation that disrupts the phototransduction cascade (*norp^A*) normal entrainment may be disrupted, but these doubly-mutant flies may still entrain to a light-dark cycle after many cycles. Only in flies carrying mutations at the *glass* locus as well as *cry^b* – which lack all ocular and extra-ocular photoreceptive function – do flies become totally ‘blind’ to the entraining effects of light. Since cryptochrome occurs in the lateral neurons considered to comprise the locomotor activity pacemaker, it becomes the most important candidate for this direct entrainment pathway to the brain.

Cryptochrome is clearly on the input pathway to the central oscillator, but its expression oscillates in a circadian fashion. For this reason it may be regarded as a *Zeitnehmer* or rhythmic input to the oscillator whose function may be to stabilise environmental ‘noise’ to give a more reliable incoming entrainment signal (Roenneberg and Mellow, 2001; Chapter 16).

3. Genes on the output pathway

Studies outlined in Chapters 5 and 6 indicate a number, perhaps a large number, of independent or quasi-independent circadian oscillators in diverse insect tissues and organs. These serve a variety of circadian outputs. Here, however, we are mainly concerned with the ‘central’ brain oscillator or pacemaker, most probably located in the so-called lateral neurons (LNs; see Chapter 8) that govern the two best studied rhythms, adult locomotion and pupal eclosion. At least in part, these two overt rhythms have separate output pathways. Known output genes include *pigment dispersing factor* (*pdf*), *lark* and *takeout* (*to*), among others such

as those underlying the synthesis and/or release of developmental and metamorphic hormones (prothoracicotropic hormone, juvenile hormone etc.).

The lateral neurons (see Chapter 8) release a neuropeptide - pigment dispersing factor (PDF) - which seems to be required for the rhythm of locomotor activity in the adult fly (Renn et al., 1999). It may also be required for pupal eclosion since over-expression of its gene *pdf* disrupts rhythmic emergence of flies from their puparia (Helfrich-Förster et al., 2000). Antibodies to PDF trace arborisations from the LNs to possible target tissues (see Chapter 8). A more recently discovered output pathway gene is *takeout* (*to*) whose expression occurs in the cardia, crop and antennae (Sarov-Blat et al., 2000) and may convey temporal information for feeding and antennal rhythms (Krishnan et al., 1999). Finally, *lark* is required for rhythmic pupal eclosion (Newby and Jackson, 1993) but not for locomotor activity; it is clearly on the output pathway for the first and not the second. Its gene product LARK acts as an RNA-binding protein that cycles in abundance in some neurons of the brain and ventral ganglionic mass, co-localising with a neuropeptide CCAP (crustacean cardioactive peptide) (Gammie and Truman, 1999) in a neuroendocrine cascade controlling ecdysis behaviour (see Chapter 8). LARK expression is eliminated in *per*-null flies; in wild-type flies, however, it reaches its highest levels during the day, perhaps suggesting that it acts as a repressor of eclosion.

C. SPATIAL AND TEMPORAL CLOCK GENE EXPRESSION

The use of antibodies raised against 'clock' proteins (e.g. Siwicki et al., 1988; Hall, 1995) has indicated their wide distribution in the fly body. Most such information concerns PER and TIM and this section reviews this material very briefly. The *period* gene, for example, may be expressed at all stages of development from embryo to adult fly, and in a wide variety of tissues. Apart from 'obvious' pacemaker sites, such as the central nervous system, these tissues include *inter alia*: gut, salivary glands, gonads, compound eyes, fat body, Malpighian tubules and epidermis. Whilst expression of 'clock' genes in the brain may be clearly associated with rhythmic phenomena such as locomotion and eclosion, that in 'peripheral' tissues suggests clock-like functions in numerous sites outside the CNS (Giebultowicz, 1999, 2000; Chapter 6). Of particular interest is 'clock' gene expression in endocrine organs such as the prothoracic glands, or Dipteran ring glands (Emery et al., 1997), which suggests clock regulation of developmental and metamorphic hormones (PTTH and ecdysteroids; see Chapter 5).

In the fly brain, the so-called lateral neurons (LNs) are of particular interest (see Chapter 8). These cells express 'clock' genes, including *per*, *tim* and *pdf* (Helfrich-Förster, 1996) and probably also others such as *Clk*, *cyc*, *dbt* and *cry*. In these and other cells with a 'clock' function there is a clear temporal succession in which PER and TIM proteins are synthesised in the cytoplasm and then transported to the nucleus. This daily movement is an essential part of the negative feedback loop making up the central oscillation (see below). Some tissues, however, notably the ovary of *D. melanogaster*, do not show such a translocation of 'clock' proteins, suggesting an absence of clock-dependent processes in this particular tissue.

D. THE CURRENT MOLECULAR MODEL FOR CIRCADIAN RHYTHMICITY

The properties and spatial distributions of the above-mentioned ‘clock’ genes may now be assembled in a word model to describe a simple feedback loop governing locomotor activity and pupal eclosion in *D. melanogaster*. In this model (Fig. 4.1) transcription of *per* and *tim* (in the lateral neurons) gives rise to two proteins PER and TIM which form a heterodimer in the cytoplasm. The formation of this dimer is delayed or accelerated by the action of *doubletime* which alters PER stability. When the PER/TIM dimer is formed it undergoes nuclear entry during a specific window (Ct 19-20) and then associates with the positive transcription factors encoded by *Clk* and *cyc*. Binding to the DNA at this point removes transcriptional activation of *per* and *tim* and negative feedback is achieved. Light affects the feedback loop by degrading TIM through the agency of *cryptochrome* (and other routes), and output from the central oscillator to overt rhythms is regulated *via* the actions of ‘output’ genes such as *pdf*.

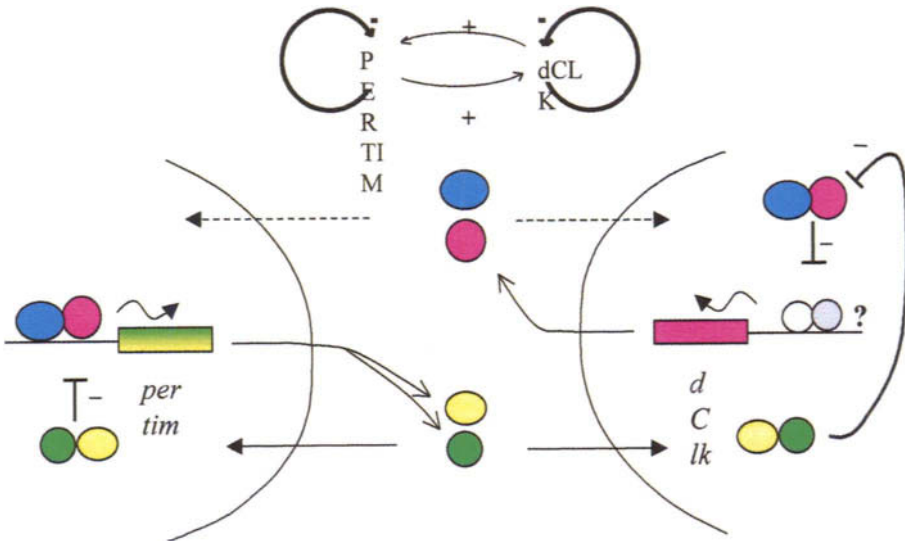


Fig. 4.1. Interlocked feedback loops in *Drosophila melanogaster*. For ease of description some elements of the *Drosophila* clockworks, whose functions are less clear, are not depicted. Left: CLK (purple) and CYC (blue) directly activate *per* and *tim* transcription in a rhythmic fashion (~). Then the PER (green) and TIM (yellow) proteins move back into the nucleus as a dimer and repress their own production. Right: The transcription of *Clk* is regulated by unknown transcription factors (?), which are inhibited by the CLK/CYC dimer. It is not known how CLK and CYC enter the nucleus, and this is indicated by dashed arrows. The PER/TIM complex represses CLK/CYC and therefore acts as a positive factor in the *Clk* negative loop. (From Rosato and Kyriacou, 2001).

E. POSSIBLE EXPLANATIONS OF CANONICAL CIRCADIAN PROPERTIES

A good model for the circadian feedback loop should be capable not only of predicting oscillation and how it occurs, but also providing a sound basis for the oscillation’s circadian

period, its temperature compensation, and its entrainment to match environmental periodicity by responding to light (and temperature) signals. The molecular feedback loop outlined above offers at least some clues to these phenomena.

1. Circadian period

The current molecular model for the 'clock' feedback loop gives few clues as to how the circadian (near 24 hour) period is achieved. Theoretical studies on feedback loops (Chapter 7; Lewis et al., 1997), however, strongly suggest that a *time delay* in the loop is required for oscillation to occur. This is provided, at least in part, by the fact that translation inevitably follows transcription; that is, the peak of protein occurs several hours after peak mRNA production. Moreover, one might expect that a short-period mutant such as *per^S* should have a shorter time delay within its loop than a long period mutant such as *per^L*. These differences in time delay may be generated by differential phosphorylation of the PER protein, as controlled by *doubletime* which, in two mutants alleles, *dbr^S* and *dbr^L*, shorten and lengthen circadian period respectively. In this respect, Goldbeter (1995) has tentatively suggested a qualitative model based on the step-wise phosphorylation of PER to produce such time delays in the loop.

Alternatively, or in addition, variations in circadian period may be generated by cell-cell coupling within a multicellular and therefore multioscillator 'clock'. In this view, detailed elsewhere (Chapter 6), tight coupling could generate a short overall period, looser coupling a longer period, and very loose or no coupling an arrhythmic pattern. In a complex multicellular clock such as a group of linked lateral neurons, period-altering interactions of this nature are possible.

2. Temperature compensation

The defining circadian property of temperature compensation of circadian period (τ) remains one of the least understood aspects of the phenomenon. The problem, addressed again elsewhere (Chapter 16, for example) suggests that this is a fundamental property of circadian systems allowing them to operate as 'clocks'. However, does temperature compensation find its explanation in molecular details of the feedback loop, or in higher order cellular interactions within a tissue-based clock? One suggestion (Sawyer et al., 1997), is that temperature compensation (in *D. melanogaster*) is regulated somehow by the length of a characteristic amino acid sequence (the Threonine-Glycine repeat) within the PER protein. This sequence shows differences between latitudinal strains and therefore between flies exposed to different temperature conditions. The major variants, Thre-Gly 17 and 20, were found to be distributed in a highly significant latitudinal cline with the latter being predominant in northern Europe with its generally cooler temperatures. Although there is a clear correlation between latitude and the occurrence of these variants, it is far from clear whether such a phenomenon is causal or merely correlative. Furthermore, the situation just described is not evident in other species, and one might have expected, *a priori*, that a more fundamental, more highly conserved, phenomenon would have been behind temperature compensation. For example, other species of *Drosophila*, and other genera, may lack such latitudinal variation and, indeed, even lack such a characteristic repeat sequence. Nevertheless, the question whether temperature compensation arises from the molecular

architecture of 'clock' genes remains a real possibility. On the other hand, as with the problem of circadian period itself, temperature compensation may be a result of higher-order interactions (e.g. cell-cell coupling) within a more complex multicellular structure making up the circadian clock. Clearly, these problems need to be resolved.

3. Phase resetting by light

In formal models (see Chapter 7), light falling in the early subjective night, during the upswing of a hypothetical light-sensitive protein, causes it to be destroyed. When darkness resumes, so does synthesis of this protein; the system thus shows the characteristic phase delay ($-\Delta\phi$) generated by illumination at this phase. Light falling later in the subjective night causes a similar destruction of the protein, but this time leads to a phase advance ($+\Delta\phi$).

In *D. melanogaster* the role of this hypothetical protein seems to be taken by TIM (see above) which is rapidly degraded by light. However, in this species (and probably other 'higher' flies) there are multiple input pathways (section B2; Chapter 8): through the compound eyes, through the H.-B. eyelet, and directly to the lateral neurons of the brain. Light cycles perceived through the compound eyes will entrain, but only slowly, taking a number of cycles before all transients subside. Light pulses arriving directly to the brain, on the other hand, cause almost 'instantaneous' phase shifts (see Chapter 3) and therefore rapid entrainment. These differences were already noted by Pittendrigh (1981) and other chronobiological pioneers as 'continuous' and 'discrete' entrainment, respectively. 'Continuous' entrainment may now be interpreted as the slow access of light through the agency of the photopigment rhodopsin in the eye (and H.-B. eyelet?), whereas 'discrete' entrainment is effected through the agency of cryptochrome in the lateral neurons. In both cases these signal pathways reach the 'clock' protein TIM occupying a central role in the feedback loop.

ANNOTATED SUMMARY

1. Initiated by the seminal paper on the *period* gene in *Drosophila melanogaster* (Konopka and Benzer, 1971), three decades of intensive research into the genetics and molecular biology of the circadian system in this 'model' organism has led to an enormous outflow of information allowing us to start opening the 'black box' of the circadian phenomenon.
2. A large number of genes are expressed in a circadian fashion, but only a select group of about five, commonly called 'clock' genes, are thought to play a central role in the auto-regulatory feedback loop making up the 'central' oscillator. Other genes play roles in the input or entrainment pathways, and others in the output pathways to the overt rhythms the central oscillator controls.
3. Crucial 'clock' genes making up the central feedback loop include *period*, *timeless*, *Clock*, *cycle* and *doubletime*. On the input pathway are genes encoding for rhodopsin (compound eyes) and for the photopigment cryptochrome (central brain). Output pathway genes include *pigment dispersing factor*, *take-out* and, for eclosion, *lark*.
4. The 'central' clock genes appear to be part of an overall negative feedback loop in which transcription of *per* and *tim* results in their proteins accumulating in the cytoplasm during the early subjective night. The PER and TIM proteins lack conventional DNA-binding sequences, but form mutual PER-TIM heterodimers by association through protein-protein

sequences called PAS domains. The PER-TIM dimers are able to re-enter the nucleus later in the subjective night, undergoing further binding to the proteins CLOCK and CYCLE which have characteristic (bHLH) DNA-binding sites, as well as PAS domains, allowing DNA binding at an E-box, thus closing the circadian loop. Time-delays in the loop, giving rise to the characteristic near-24 hour period, and perhaps also to short and long *per* phenotypes, may be effected by *doubletime*, encoding a protein kinase that shortens or lengthens circadian period by differential phosphorylation of PER.

5. Genetical and molecular aspects of the auto-regulatory feedback loop, and of its input and output pathways, thus begin to provide explanations for the canonical properties of the circadian system - namely period length, its persistence in the absence of environmental cues, its temperature compensation, and its ability to entrain to environmental light cycles. Our rapidly advancing knowledge gained from a study of genetics and molecular biology is therefore beginning to explain many chronobiological phenomena in concrete, rather than abstract, terms. However, although enormous strides have been taken, the circadian system is a complex one, and many unanswered questions remain.

This Page Intentionally Left Blank

CHAPTER 5

PHYSIOLOGY OF CIRCADIAN SYSTEMS

By C.G.H. Steel and X. Vafopoulou,
Department of Biology, York University, Toronto M3J 1P3, Canada

Be near me when my light is low/When the blood and the nerves prick.
Alfred, Lord Tennyson

CONTENTS

Introduction	116
A. <i>Central and Peripheral Oscillators</i>	116
1. Early studies	117
2. Peripheral oscillators	117
3. Central oscillators	118
B. <i>Overview of Insect Life Cycles and their Control</i>	120
1. Types of life cycle and stage-specificity of rhythms	120
2. The classical scheme of hormonal regulation	122
3. Early evidence of rhythmicity in the neuroendocrine system	123
C. <i>Circadian Organisation in Larvae</i>	124
1. Circadian regulation of developmental hormones	124
(a) Prothoracicotrophic hormone	124
(b) Moulting hormones	130
(c) Multioscillator organisation of developmental hormones	135
2. Ecdysis	138
3. Rhythms in cuticle formation	147
4. Egg hatching	152
D. <i>Circadian Organisation in Adults</i>	156
1. Circadian regulation of adult hormones	156
2. Gamete formation and transport	161
(a) Oogenesis	162
(b) Spermatogenesis	163
(c) Ovulation and egg transport	163
(d) Sperm release and transport	164
3. Chemical communication: Pheromones	168
(a) Sex pheromones	168
(b) Circadian rhythms in sex pheromones in moths	169
(c) Rhythmicity in neuroendocrine mechanisms that control sex pheromone production	172

4. Circadian rhythms in olfactory responses	176
5. Photic communication: bioluminescence	178
6. Acoustic communication: stridulation and courtship song	182
7. Mating	184
8. Oviposition	185
Annotated Summary	186

INTRODUCTION

THE focus of physiology is on the *functions* of tissues, organs and organ systems. In order to understand these functions, physiologists must have knowledge of molecular interactions and how these set the stage for cellular processes, how these processes influence the relationships of cells with one another and how interactions between groups of cells are coordinated within the whole organism. Physiology is integrative functional biology.

Circadian systems are responsible for generation and maintenance of internal temporal organisation, and are consequently a pivotal mechanism of internal coordination. Therefore, they are central to physiology. In the broad sense, all of this book addresses the physiology of circadian systems, with different chapters focussing on the different levels of physiology. The formal properties of circadian systems (Chapters 2 and 3) are studied with the same goal of understanding function and are often called 'dry' physiology, in contrast to the 'wet' blood-and-guts approaches of more familiar physiology (Menaker, 1978). This chapter focuses on physiology at the level of intercellular communication and coordination and the operation of these mechanisms within the whole insect.

No comprehensive review of the physiology of circadian systems has been assembled since Page (1985). Excellent detailed reviews are available on specific areas of this subject, as the sections that follow recognise. The present treatment reveals some features of the physiology of rhythmic systems that are not apparent from specialised reviews. First, there are numerous areas of insect physiology for which control mechanisms have been reported in detail but which have yet to gain the attention of circadian biologists; equally there are many described circadian phenomena whose physiological control is largely unknown. Both these observations are especially applicable to the many aspects of reproduction, where circadian physiologists will find a gold mine of research opportunities. Second, the importance of the neuroendocrine system is paramount; it constitutes not only diverse output pathways that convey temporal information to target tissues, but is now seen as an integral part of the timekeeping system itself. Finally, the realisation is emerging that many independently described rhythmic phenomena are regulated by common sets of controls. Central among these controls are the hormones that regulate development and reproduction; circadian systems that control these hormones are becoming clear, perhaps revealing a central timing system.

A. CENTRAL AND PERIPHERAL OSCILLATORS

Detailed evidence of the presence of numerous circadian oscillators in various anatomical loci in insects is given elsewhere (Chapters 4 and 8). The purpose of the present section is to discuss the ramifications of this information at the level of physiology. In particular, the physiologist is concerned with the functional significance of these various oscillators, and the mechanisms whereby multiple oscillators are coordinated within the organism.

1. Early studies

The early studies of clock localisation and function in insects employed traditional physiological techniques such as surgery and transplantation. These studies followed a uniform sequence of experimental steps. First, an overt rhythm (e.g. a behaviour) would be characterised and certain formal properties of the rhythm examined. This information enabled the affects of ablation of candidate photoreceptors (such as eyes, ocelli, brain) to signal the site of light input, generally as the structure whose elimination resulted in a free-running rhythm (see Chapter 8). Then, structures whose removal caused arrhythmicity were sought. Arrhythmicity can result from either removal of an oscillator locus or from interception of the output pathway to the overt rhythm. Therefore, several sites would commonly be found whose removal led to arrhythmicity. The oscillator locus was therefore tentatively assigned to the site necessary for rhythmicity that was closest to the photoreceptors. *In vitro* studies of rhythm generation by the isolated candidate oscillator would confirm its ability to generate a circadian rhythm. Nervous and/or hormonal output pathways to the overt rhythm could then be examined by transplantation and ablation experiments. The physiological approach to oscillator location requires knowledge of both the driven rhythm and the entrainment pathway. The identified oscillator will therefore qualify as a circadian clock in the broad biological sense (Chapter 1). Clocks localised by this approach are among the best known and most fully characterised in insects (see Chapter 8) precisely because the design of these kinds of experiments requires extensive prior knowledge of the properties of the clock. However, these techniques do not allow resolution of the location of the oscillator to anything smaller than a lump of tissue, potentially containing hundreds of cells.

2. Peripheral oscillators

During the 1990s, a group of functionally related genes was cloned from *Drosophila* which are understood to participate in the core mechanism that generates circadian oscillations within cells (see Chapter 4). The technical ease of measurement of the RNA and protein derived from these 'clock genes' has resulted in the discovery of many novel loci of cyclic gene expression. It has been convenient to refer to such cells as 'clock cells', but it should be borne in mind that, in most cases, these cells are known only to possess one or two cogs of a molecular oscillation, which is insufficient evidence to show that the cells have a functional role in timekeeping. Most of these loci have yet to be the subject of functional analysis and their contribution to circadian organisation is unknown. Some of these loci will be found to be important loci of circadian oscillators and possibly clocks. The mechanisms by which these novel circadian loci are coordinated and how they communicate with one another will be an important emerging area of circadian biology. But some of these loci may be found to have quite different functions. Indeed, it has never been shown that expression of 'clock genes' occurs *only* in 'clock cells'; it remains possible that these genes are expressed in cells with no role in timekeeping. This possibility is fortified by the finding in many cells of extremely high levels of 'clock gene' transcripts or proteins, well above levels associated with transcriptional regulators. PER and TIM (see Chapter 4) are routinely detected in cells at levels comparable to those of secretory proteins and may be found at great distance from the nucleus (Sauman and Reppert, 1996b). It has therefore been suggested that these proteins may be multifunctional, implying that they may be present in cells with no molecular oscillator. Functions of 'clock genes' in non-circadian phenomena include cocaine sensitisation (Andretic et al., 1999) and the

ultradian male courtship rhythm (Hall, 1996). It is also possible that 'accidental leakage of gene expression' (Dow and Davies, 2001) may occur.

The above considerations show that it is imperative that potential 'clock cells' which have been identified by the presence of cycling clock proteins or mRNAs are examined for the presence of rhythmic outputs; in other words, are these cells engaged in circadian control? Evidence from mammalian systems indicates that the answer will be complex. Work with cell lines of the suprachiasmatic nucleus (SCN) has shown that these cells are able to communicate rhythmicity to other cells (Allen et al., 2001), confirming their importance in circadian regulation. Fibroblasts (Yagita et al., 2001) and smooth muscle cells (Nonaka et al., 2001) can exhibit circadian expression of the same 'clock genes' as SCN cells, but expression is both induced (Nonaka et al., 2001) and phase-set (McNamara et al., 2001) by hormones. Further, these induced 'clock gene' oscillations failed to drive rhythmicity in other cells. The emerging picture from mammalian research is that peripheral oscillators are cells that exhibit circadian expression of 'clock genes', but this rhythmicity is driven from without and is not transmitted to other cells. Accordingly, peripheral oscillators are not clocks (Allen et al., 2001). As noted by McNamara et al. (2001) and Herzog and Tosini (2001), hormones appear to play a crucial role in coordinating peripheral oscillators.

In insects, peripheral oscillators have been studied primarily in a transgenic strain of *Drosophila* in which expression of a luciferase gene is driven from the promotor of the *per* gene (see Chapter 4). Numerous epidermal tissues (Plautz et al., 1997) and Malpighian tubules (Hege et al., 1997) were found to glow rhythmically. These rhythms could be reset by light in large pieces of the animal (e.g. a leg), showing that these peripheral oscillators are light entrainable. This photosensitivity was examined in Malpighian tubes (Giebultowicz et al., 2000). *In vivo*, the *amplitude* of the rhythm was light sensitive, but it is not known if light can phase shift the rhythm *in vitro*. The existing data suggest that entrainment requires intact connections between the tubule and the rest of the animal. It is not known if these rhythms have a functional significance or whether rhythmicity can be transmitted between cells. Therefore, it is not yet known whether these peripheral oscillators qualify as clocks. These uncertainties concerning insect peripheral oscillators (photosensitivity, persistence of rhythmicity, function) can all be resolved when 'clock gene' expression is studied in insect cell lines. However, there are two peripheral oscillator loci in insects that do qualify as clocks. The chemosensory hairs of the antennae of *Drosophila* exhibit circadian expression of *per* (Krishnan et al., 1999; see section D4), and this is necessary for the circadian rhythm of olfactory responsiveness; the antennal oscillator qualifies as a peripheral clock. Also, the male reproductive system of several moths contains a photosensitive circadian oscillation that times the rhythmic movement of sperm along the duct system (details in Section D2d). This also is a peripheral clock. The prothoracic gland oscillator (Section C1b) is peripheral in location, but not in function. It is a component of a multioscillator timing system that may be a central clock of the insect (see below). The gland generates a light entrainable circadian rhythm of hormone release *in vitro* (Vafofoulou and Steel, 1998), so it also is a clock.

3. Central oscillators

'Clock gene' expression has been found in various parts of the central nervous system but the question of function has been investigated in very few of these. The most extensively studied are the 'lateral neurons' in the brain of *Drosophila*, which seem to regulate the circadian rhythm of locomotion (see Chapter 4). Later sections of this chapter describe various loci of the CNS implicated in circadian control of specific rhythmic processes, most of which

have yet to benefit from studies of 'clock gene' expression. Readers of insect circadian biology could readily acquire the impression that insects possess a multitude of potential circadian oscillators, each of which appears to control one phenomenon, is entrained to the outside world independently of the others and seems not to interact with the others. Such an impression reflects the historical progression of the subject, which has reached a point of documenting numerous phenomena that are under clock control but is only beginning to grapple with the mechanism by which the component clocks of an organism are coordinated. There are many reasons to both expect and require integration within the circadian system of an insect, of which a few deserve comment.

Insect researchers have traditionally viewed their experimental animals as 'simple' organisms, which presumably operated by simple rules; this view inhibited recognition of complex regulatory mechanisms such as the integration between multiple timing systems. Many of the functional interactions are of a level of complexity comparable to vertebrates (for an early discussion, see Steel and Davey, 1985). An organism that was constructed from a host of autonomous clocks would possess maladaptive rigidity. All complex animals are constantly responding to a diversity of changes in both the internal and external environments; these responses involve subtle adjustments to numerous internal systems. Given that the maintenance of internal temporal organisation in a tempestuous world is critical, even paramount (see Pittendrigh, 1993), continuous and integrated adjustments within the circadian system are essential. This line of reasoning has led to the invocation of a 'master clock' in charge of the clock shop. Reality likely lies in the middle ground; a single master clock is too imperial to be adaptive just as a host of autonomous clocks is too inflexible to be adaptive. The middle ground has been articulated very clearly in various vertebrate species, including reptiles (Tosini et al., 2001), birds (Nichelmann et al., 1999) and mammals (van Esseveldt et al., 2000). In all these systems, there is no discrete 'master' clock. There is, however, a system of coupled oscillators that collectively are able to communicate temporal information throughout the organism and thereby to maintain temporal organisation in response to both internal and external changes. These integrated oscillators are found in the SCN, ocular retina, pineal organ and parietal eye, according to species. Together, these oscillators form a central timing system, or central clock. An important feature of known systems is that each constituent oscillator is responsive to a particular variety of stimuli and has outputs that employ different pathways from the others. Coupling results from the fact that outputs of one act as inputs to the others. The term 'clock' is essentially a network property of the population of oscillators and not exclusive to any one. By definition, a central clock must be able to regulate the timing of fundamentally important events in the organism. In order to do this, it must have outputs to numerous and diverse targets in which it drives rhythmicity. If the clock has outputs that are both neural (for local rhythms) and hormonal (for regional or general rhythms), it can potentially regulate rhythmicity in any part of the organism. This last consideration suggests why it may be that vertebrate central clocks all have both neural and endocrine components. The above principles of functional organisation are as applicable to insects as to vertebrates.

Is there a central clock in insects? Clues that there is come from both anatomical and functional lines of evidence. A region of the insect brain that is analogous to the vertebrate SCN has been known for many decades but has received attention from circadian biologists only recently. First, early studies of the anterior pituitary and corpus allatum revealed that they share remarkable embryological similarities. Both develop from a local inpushing of the stomodaeum and both structures subsequently become associated with an outgrowth of the brain, which is known in vertebrates as the posterior pituitary and in insects as the corpus cardiacum (Scharrer and Scharrer, 1944). Second, the nerve terminals in the posterior pituitary

derive from cell bodies in the hypothalamus, exactly as the nerve terminals in the corpus cardiacum in the dorsal protocerebrum (Hanström, 1939). Third, these two embryologically analogous brain regions both differentiate into a cluster of peptidergic neurosecretory cells with similar functions. In both systems, these peptidergic cells synthesise and release a diversity of hormones that regulate growth, reproduction and various aspects of metabolism. In sum, there is impressive analogy in embryology, anatomy and function between the pituitary and the corpus cardiacum-allatum complex of insects and between the hypothalamus and dorsal protocerebrum of insects. Are there 'clock cells' within the dorsal protocerebrum, analogous to the SCN in the hypothalamus? Evidence is accumulating that the dorsal protocerebrum contains a group of 'clock cells' (Terry and Steel, 2002) that regulates rhythmic release of the neuropeptides PTTH (Vafopoulou and Steel, 1996a) and bombyxin (Vafopoulou and Steel, 2002) among others (unpublished observations) and probably also JH in the corpus allatum (see Section D1). These 'clock cells' also participate in the photoperiodic control of release of PTTH (Sauman and Reppert, 1996a) (for details, see Section C1a). These 'clock cells' are not only adjacent to the peptidergic cells, but are also adjacent to the corpora pedunculata (mushroom bodies) which integrate sensory input with behaviour (Schurmann, 1987). Thus, they are strategically located to exert circadian control over numerous hormones and over the neural pathways of behaviour. Therefore, this group of 'clock cells' is closely analogous to the SCN both in location relative to their outputs, and in function. The analogy with vertebrate systems is therefore extending beyond neuroendocrinology and into the neuro-architecture of circadian control. These insect 'clock cells' clearly have all the prerequisites of a central oscillator since they have the required access to circadian control over myriad physiological processes. This putative central oscillator is part of a multioscillator system that includes the prothoracic glands and possibly other loci that are required for further dissemination of temporal information to target tissues, much as the SCN is coupled to other oscillators. It is possible that the complete insect central clock will comprise all these oscillators. Much remains to be elucidated about this putative central clock system and its integration with peripheral oscillators, but numerous clues will be discerned in the sections that follow.

B. OVERVIEW OF INSECT LIFE CYCLES AND THEIR CONTROL

1. *Types of life cycle and stage-specificity of rhythms*

Insects pass through a series of distinct stages prior to reaching the adult form. The number of these instars is usually constant for a species. When food is plentiful, each instar is larger than the preceding one. The insect exoskeleton is relatively inelastic and so limits the amount of tissue growth that can occur inside. The entire exoskeleton is replaced at intervals; a new and larger one is formed beneath the old one under the control of hormones. This process is known as moulting. When the new exoskeleton is sufficiently formed, the insect escapes from and discards the old exoskeleton. The behaviours by which this is achieved are termed ecdysis. The timing of ecdysis is therefore closely tied to the process of moulting (see Section C2). The new exoskeleton usually displays morphological differences from the old one. Similarly, the soft tissues within the insect also undergo degrees of reorganisation during moulting. These simple facts are important in the context of the physiology of circadian rhythms because they emphasise that each instar possesses a unique set of equipment, both morphological and anatomical with which to generate and express circadian rhythms. Consequently, each instar has a unique circadian repertoire. The extent to which the repertoire

of one instar differs from that of the other is a function of the extent of reorganisation that occurs in the insect during moulting. This reorganisation is known as metamorphosis. Thus, moulting is accompanied by a degree of metamorphosis. Insects are grouped according to the magnitude and temporal pattern of metamorphosis in the life cycle.

In wingless insects (Apterygota), each instar is morphologically very similar to the others, the only clear metamorphosis being maturation of the reproductive system in the penultimate instar. These are (incorrectly) termed ametabolous insects. Little is known of circadian rhythms in these insects. In hemimetabolous insects, the immature stages (larvae) bear some morphological resemblance to the adult stage. At each moult a degree of metamorphosis occurs towards the adult form; the larval-adult moult involves substantial metamorphosis including maturation of the reproductive system, completion of development of the flight apparatus (wings, muscles etc) and reorganisation of sensory receptors (e.g. the appearance of ocelli) and associated remodelling of the nervous system. Hemimetabolous insects whose circadian systems are well studied include cockroaches, crickets and bugs such as *Rhodnius prolixus*. In holometabolous insects, the larval instars resemble worms or caterpillars. In addition to this radical difference from the adult form, these larval stages also differ in morphology from each other. The last larval stage moults into a pupa, which is sedentary and cannot feed, but has developed numerous adult features. Completion of metamorphosis into the adult form occurs within the pupa. The ecdysis of the adult from a pupal case is known as eclosion. Insects with this type of life cycle that are familiar to circadian biologists include moths (such as *Antheraea*, *Bombyx*, *Heliothis*, *Manduca* and *Samia*) and flies (such as *Drosophila*, *Musca*, *Calliphora* and *Sarcophaga*).

From the above, it should be apparent that each instar of an insect is to some degree morphologically and anatomically different from the others, creating a unique set of circadian machinery. There are visible changes in the structures (e.g. appendages) with which circadian rhythms in behaviour can be expressed, but also less visible changes in the musculature available to move them, the nervous pathways and motor programmes that determine the type of movements, the sense organs available to synchronise internal clocks with environmental *Zeitgeber*. Even the number and variety of 'clock cells' within the animal changes between instars (see Chapter 4). Further, superficially similar overt rhythms may occur in different instars, but are achieved by different neurophysiological equipment and have different functional significance to the insect. For example, a locomotor rhythm is seen in almost all instars but is regulated and performed by radically different nerves and muscles in larvae and adults (e.g. foraging for food may occur in both larvae and adults but walking and flying employ quite different structures and control pathways). The functional significance of a rhythm may vary even within an instar. For example, locomotion in the adult may at times be associated with foraging and other times with mate-seeking. Are the underlying control systems the same when the function is different? Thus, the expression of circadian rhythms is markedly stage-specific.

This stage-specificity is itself a consequence of the mechanisms that regulate insect development. These mechanisms are primarily hormonal. Thus, developmental hormones determine the circadian equipment available to each instar. Recent evidence shows that the converse determination is also true; key components of the circadian system regulate rhythmicity in the developmental hormones (see Section C1). Consequently, the circadian system is intertwined with development throughout the life cycle.

2. The classical scheme of hormonal regulation

The endocrinology of insect moulting and metamorphosis are articulated in standard reviews (Steel and Davey, 1985; Wigglesworth, 1985; Gilbert et al., 1996). This section presents a brief overview of this scheme, with emphasis on those elements relevant to circadian rhythms.

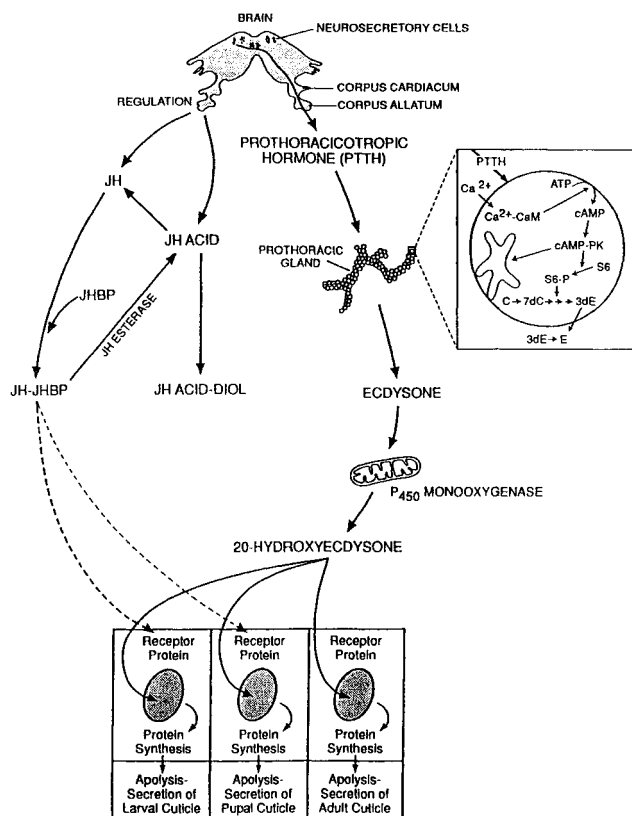


Fig. 5.1. The classical scheme of neuroendocrine control of insect moulting and development. Environmental cues intermittently trigger release of the brain neuropeptide prothoracicotropic hormone (PTTH). PTTH activates synthesis and release of ecdysone by the prothoracic glands (via a complex biochemical pathway; inset). It is assumed that ecdysone synthesis requires initial activation by PTTH but not continued regulation. Ecdysone is converted to 20-hydroxyecdysone (20E) by P₄₅₀ monooxygenase. 20E acts on target tissues (e.g. epidermis) via a steroid receptor system where it elicits specific cellular responses essential to development. The release of juvenile hormone (JH) from the corpus allatum plays a modulatory role on the responses of the target tissues to 20E, thus determining the quality of a moult, i.e. larva-larva, larva-pupa or pupa-adult. (From Gilbert et al., 1996).

The central elements of the 'classical scheme' had been elaborated by the early 1950s, using techniques such as decapitation, organ transplantation and parabiosis. Studies of the physiology of circadian regulation only became feasible many decades later, when techniques

were developed that would measure amounts of the hormones involved. These studies showed that the brain was necessary for moulting for the first few days of each instar, during which time it released a hormone required to enable the prothoracic glands (PGs) to synthesise the steroid moulting hormone, *ecdysone* (see Koolman, 1989). The brain hormone became known as *prothoracicotrophic hormone* (PTTH) a neuropeptide (see Ishizaki and Suzuki, 1994). After the first few days of the instar, the process of moulting no longer requires the head. In a population of insects, the transition to head-independence occurs over a period of time (often about two days) called the *head critical period* (HCP). After the HCP, the production of moulting hormone by PGs progressively increases, reaches a peak and then decreases, causing parallel changes in the haemolymph hormone titre (see Steel and Vafopoulou, 1989). Moulting hormone acts on ecdysone-responsive genes in target tissues (Cherbas and Cherbas, 1996) whose expression is manifest as moulting. This classical scheme envisages the sequential action of PTTH and moulting hormone as an 'endocrine cascade' (Gilbert et al., 1996) (see Fig. 5.1). The degree of metamorphosis that accompanies moulting is regulated by the levels of *juvenile hormones* (JHs) synthesised by the corpora allata at particular times in the instar (Fig. 5.1).

3. Early evidence of rhythmicity in the neuroendocrine system

The first line of evidence indicating rhythmicity in the insect neuroendocrine system came from cytological observations of daily cycles in the appearance of various cells or cell types. These findings were important because they revealed the existence of rhythmicity and so led to later, more detailed studies. They are limited by the fact that almost all reports merely described correlations between rhythmic events such as cycles in cells and rhythmic behaviour. Experimental demonstration that a particular rhythm of cellular events was responsible for any other rhythm was seldom attempted. Consequently, there is a general absence of information distinguishing cause from effect in this literature. The seriousness of this limitation became apparent in the 1960s, when it became clear that cellular rhythmicity was the norm rather than the exception, prompting Brady (1967) to observe, "the fact that synchronous changes take place in the nuclei of widely different tissues, varying from fat body to neurosecretory cells, tends to suggest that one of the effects of the fundamental circadian organisation of organisms may be to produce changes in all, or many, of their cells".

One of the first informative cytological studies conducted on the beetle *Carabus nemoralis* (Klug, 1958), found diurnal changes in the relative numbers of two types of 'active' neurosecretory cells in the brain, and also in the nuclear diameters of the cells in the corpora allata. Beck (1964) studied the diameters of the neurosecretory cells of diapausing larvae of the European corn borer, *Ostrinia nubilalis*, maintained in LD 12:12, and described a "well-defined" trimodal rhythm with maxima at 0, 8 and 16 hours after the onset of light. One peak coincided with dawn itself and was apparently phase-set by the light-cycle. However, cell sizes varied widely, and Brady (1967) has drawn attention to the possible errors which may arise in measurements of this kind.

Cymborowski and Dutkowski (1969,1970) demonstrated daily cycles of RNA and protein synthesis in the neurosecretory cells of the brain and the suboesophageal ganglion of *Acheta domesticus* by autoradiographic methods. In LD 12:12, RNA synthesis in the brain neurosecretory cells was at its maximum during the period of least locomotor activity, 30 minutes after lights-on. Conversely, it was at its lowest when the crickets were most active; the two maxima were about 12 hours apart. In the suboesophageal ganglion, on the other hand, intensified RNA synthesis occurred at two points with the first peak about 6 hours before

maximum locomotor activity. The synthesis of protein and the accumulation of neurosecretory material were also cyclic (Cymborowski and Dutkowski, 1970) and a temporal and presumed causal sequence of RNA synthesis, protein synthesis and activity was postulated. These authors and their associates also found cyclical changes in acetylcholinesterase activity (Cymborowski et al., 1970) and in the ultrastructure of the medial neurosecretory cells of the brain (Dutkowski et al., 1971). These changes were not apparent in LL (which suppresses locomotor activity in *Acheta*); the persistence of these rhythms in DD was not investigated.

Heinzeller (1976) measured neurosecretory 'activity' in the pars intercerebralis and corpora allata of the honey bee, *Apis mellifera*, and found minimal secretion at midday. One of the most extensive investigations of this type was carried out with *Drosophila melanogaster*. These studies revealed daily bimodal changes in the nuclear sizes of corpus allatum and pars intercerebralis in the adult (Rensing, 1964), and similar bimodal cycles of mean nuclear size in the corpus allatum, fat body, prothoracic glands and brain neurosecretory cells in the larva (Rensing et al., 1965). These cyclical changes were measured in LD 12:12 and were considered to reflect metabolic activity. In the salivary glands these rhythms continued in *in vitro* preparations for 10 days, and also persisted in LL for at least 4 days (Rensing, 1969). The phases of the peaks could be shifted during larval development by a 6-hour photoperiod, or by the *in vitro* addition of ecdysone (Rensing, 1971). Such preparations drew attention to the idea that timed secretion by the brain could be a key event regulating the timing of subsequent endocrine changes and the imposition of rhythmicity on target cells (see Section C1b).

C. CIRCADIAN ORGANISATION IN LARVAE

1. Circadian regulation of developmental hormones

(a) Prothoracicotropic hormone (PTTH)

The first experimental evidence of circadian involvement in insect development came from findings that the 'head critical period' (HCP) for moulting was a gated event (Truman, 1972). Neck ligation experiments showed that larval moulting in the tobacco hornworm *Manduca sexta* becomes independent of the head in one of two consecutive scotophases. This independence of moulting from the head has been widely interpreted as an indication that the PGs have been 'activated' sufficiently by PTTH from the brain to sustain ecdysteroid synthesis without further stimulation (see Bollenbacher and Granger, 1985). During the 1970s and 1980s the HCP was widely assumed to provide an indication of the timing of PTTH release. Subsequent measurements of PTTH release and its levels in haemolymph, however, have revealed a different picture. Nevertheless, the fact that the HCP is gated constitutes evidence of clock control over some key endocrine event(s) during development.

During the moult to the pupal stage, larvae of *Manduca* undergo an abrupt transition in appearance and behaviour. Within a few hours, feeding ceases, the gut is evacuated ('gut purge'), the heart becomes visible through the dorsal cuticle and pink pigment appears along each side of the heart. These *prodroma* (signs) of impending pupation are also gated (Truman and Riddiford, 1974) and exhibit a HCP for their occurrence which occurs about two days earlier than the HCP for the pupal moult (Truman and Riddiford, 1974). Which gate will be used by an individual insect is determined by the day on which it reaches a critical weight of 5g (see Fig. 5.2). These early studies established that head-centred mechanisms (presumably PTTH) impose very precise temporal organisation on a variety of developmental events.

The experiments of Truman were repeated on the commercial silkworm, *Bombyx mori*, with only quantitative differences in the results (Sakurai, 1983; 1984). More detailed results

were obtained in another silkworm, *Samia cynthia*, in which the HCP for larval moulting is sensitive to phase-shifts, and gating of the HCP persists in DD but is lost in LL (Fujishita and Ishizaki, 1981); the HCP for gut purge at the pupal moult is also phase-shiftable (Fujishita and Ishizaki, 1982). These data added to the accumulating evidence of circadian control. Fujishita and Ishizaki (1982) emphasised that the HCP is a poor indicator of the time of PTTH release, noting release could start before it and/or continue after it. Similarly, Sakurai (1983) noted that if the single release of PTTH he detected by neck ligation was repeated in consecutive scotophases, this would not have been detectable. It must be recalled that the HCP for moulting 1) signals the time after which the PGs are fully activated and as such signals a change in the secretory behaviour of the PGs, not a change in PTTH, and 2) is a population measurement from which the duration of events in individuals cannot be inferred (compare with the gating of eclosion in Chapter 3). The duration of the gate for eclosion is not a measure of the duration of eclosion behaviour in individuals. Collectively, these early studies emphasised the need for caution in interpreting experiments in which no hormones are actually measured, and the need for development of techniques for quantification of the hormones involved.

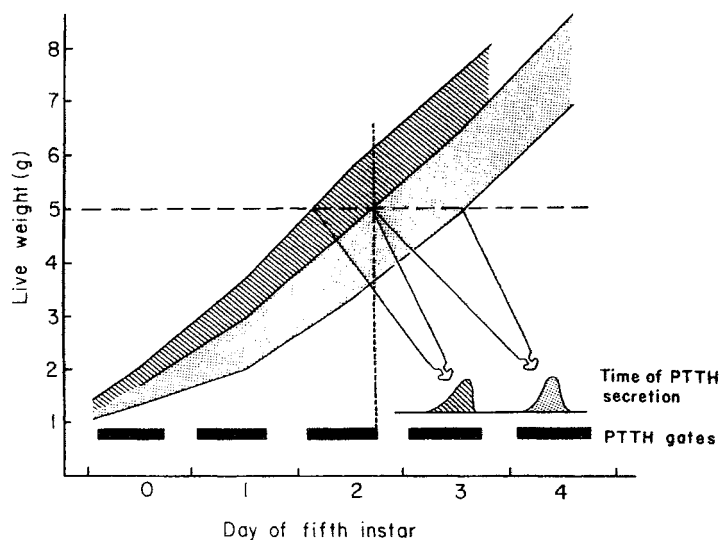


Fig. 5.2. Neck ligation experiments reveal a relationship between weight gain and HCP (the presumed time of PTTH release) in the fifth larval instar of the tobacco hornworm, *Manduca sexta*. Larvae which attain a critical weight of 5g on day 2 (hatched area) undergo HCP on day 3. Larvae which obtain this critical weight on day 3 (stippled area) undergo HCP on day 4. (From Nijhout, 1981).

Bollenbacher et al. (1989) showed that PTTH was found in the haemolymph during the scotophase of gate I, day 1, fourth instar *Manduca* and absent during the preceding and following photophases, thereby supporting the concept of a gated PTTH release. Unfortunately, no data were obtained for consecutive scotophases or for gate II or III larvae, so it is unknown whether this release is repeated. Shirai et al. (1993) were the first to assay PTTH release from brains throughout an instar, using fifth instar *Bombyx mori*. PTTH release was detectable in all but three of the 24 time-points assayed during the twelve days of the instar, both before and after the HCP for moulting. The authors reported their data as five separate

periods of release during the instar. Close examination of the data of Shirai et al. (1993) reveals that the release of PTTH by brains during the scotophases invariably exceeded release during the next photophase, with the sole exception of the day on which the animals were artificially 'resynchronised' at the onset of wandering behaviour. Seven of the 10 peak values reported were from scotophase brains. Release of PTTH throughout both the fourth and fifth instars of the same strain of *Bombyx* was described using a fluoroimmunoassay (FIA) for PTTH by Dai et al. (1995). It was again found that PTTH was released every day of both instars with differences between photophase and scotophase visible in the data. A later study using FIA for PTTH in *Bombyx* revealed PTTH in the haemolymph on every day of the fourth, fifth and pupal stages (Mizoguchi et al., 2001). In *Rhodnius*, a conspicuous daily rhythm of release of PTTH from the brain was found throughout larval-adult development (Vafopoulou and Steel, 1996a). In *Periplaneta americana*, PTTH release was found for at least three consecutive days during the week after the HCP (Richter, 2001). Collectively, these reports show that PTTH is found throughout long periods (perhaps all?) of an instar. PTTH release is clearly not confined to the HCP and therefore the notion that the HCP represents the time of PTTH release is not correct. Sakurai et al. (1998) reached the same conclusion from analysis of the timing of ecdysteroid-dependent developmental events in fifth instar *Bombyx*, rather than measurements of PTTH levels. Sakurai et al. (1998) concluded that the HCP represents the time during which PGs develop increased responsiveness to PTTH and target cells become more responsive to ecdysteroids. These events do not require any change in PTTH release. It should be emphasised that the gated nature of the HCP and its definition are not in question, and it remains clear that a profound clock-controlled event occurs at this time during development. The precise nature of this event remains unclear.

Clear evidence of sustained, rhythmic release of PTTH was obtained using *Rhodnius prolixus* (Vafopoulou and Steel, 1996a). *Rhodnius* is well suited for studies of rhythmicity during development for several reasons. First, the long duration of the instar (21 days for larval-adult development) permits the detection of circadian changes in hormones unobscured by concurrent developmental changes in hormone levels. Second, development is initiated by a single large blood meal, which enables the initiation of development in a population of insects to be synchronised to the minute. Third, this initial synchrony is maintained throughout development because no further food intake occurs; in most insects, rates of development depend on food intake (see Steel and Davey, 1985). Brains were removed every 6 hours for many consecutive days during larval-adult development and the amount of PTTH released *in vitro* quantified by a refined *in vitro* bioassay (Vafopoulou et al., 1996). Release of PTTH occurred daily, with peaks every scotophase (Fig. 5.3). Further, levels of PTTH in the haemolymph were also rhythmic and peaked in synchrony with release of PTTH from the brain (Fig. 5.4). The amount of PTTH in the brain also cycled, undergoing a sharp drop during the time of release each scotophase; about a third of the PTTH in the brain is released each night (Fig. 5.4), and the amount in the brain is restored (presumably by PTTH synthesis) during the photophase. Thus, synthesis, release and haemolymph levels of PTTH are all rhythmic. In *Bombyx*, the haemolymph titre of PTTH shows a daily rhythm during the fifth instar (Dedos and Fugo, 1999) and throughout pupal-adult development (Mizoguchi et al., 2001). Rhythmic release of PTTH from the brain is also seen in *Periplaneta* (Richter, 2001).

The rhythm of PTTH release persists in DD for at least 5 cycles in *Rhodnius*, with a period length close to 24 hours that is temperature compensated (Vafopoulou and Steel, 1996b). Therefore, PTTH release from the brain is under circadian control. In LL, the PTTH release rhythm adopted a shorter period length and damped after 3 to 5 cycles (Vafopoulou and Steel, 1996b). When arrhythmic animals that had spent 30 days in LL (Fig. 5.5a) were

transferred to DD, a free-running rhythm of PTTH release was initiated (Fig. 5.5b). Thus, the clock regulating PTTH release is photosensitive.

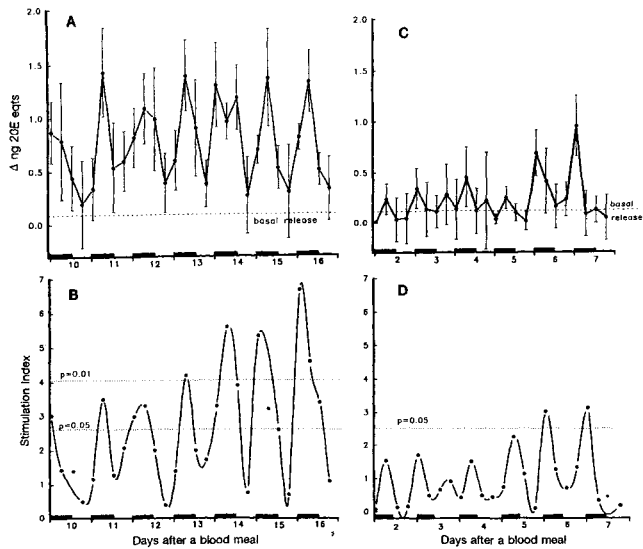


Fig. 5.3. Entrained rhythm of PTTH release from brain-retrocerebral complexes during larval-adult development (days 10-16 of the fifth larval instar) of the bug *Rhodnius prolixus* (A). The PTTH rhythm commences on day 6 (HCP)(C). The PTTH rhythm is also depicted as daily changes in the levels of a stimulation index, which is computed from the numerical values shown in Panels A and C (Panels B and D). PTTH release reaches maximum during each scotophase (dark bars) and is close to background levels (basal release) during each photophase. (From Vafopoulou and Steel, 1996a).

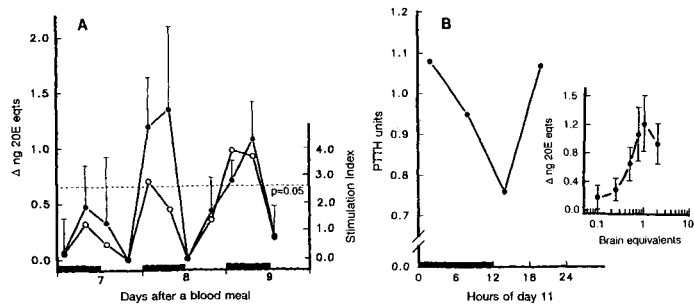


Fig. 5.4. Entrained rhythms of PTTH activity in the haemolymph (A) and brain-retrocerebral complex (B) of fifth instar larvae of *Rhodnius prolixus*. Both rhythms exhibit peaks during each scotophase (dark bars) and minima during each photophase. (From Vafopoulou and Steel, 1996a).

In *Rhodnius*, PTTH is produced by a single paired neuron in the dorsal protocerebrum (Nseiri and Steel, 2002). A group of neurons adjacent to this cell is immunoreactive for the 'clock proteins' PERIOD (PER) and TIMELESS (TIM) (Terry and Steel, 2002).

Both PER and TIM undergo circadian cycling in both abundance and nuclear migration indicating they are true 'clock cells'. These cells occupy a location in the brain equivalent to the 'dorsal neurons' of *Drosophila melanogaster*. In *Rhodnius* surgical disconnection of all

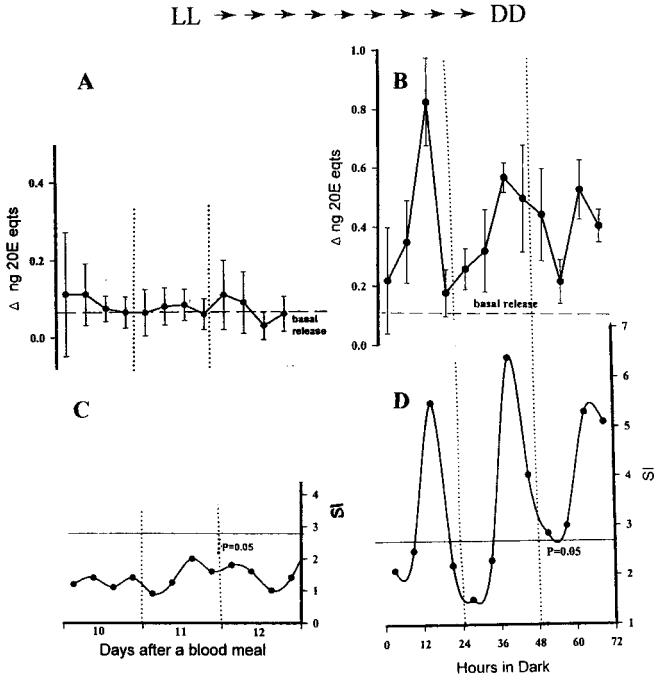


Fig. 5.5. Induction of rhythmicity of PTTH release by the brain-retrocerebral complex of fifth instar larvae of *Rhodnius prolixus* by transfer from LL (A and C) to DD (B and D). Animals maintained in LL for long periods of time exhibit arrhythmic PTTH release (A and C). A free-running rhythm of PTTH release commences after transfer. Panels C and D show the levels of statistical significance of the numerical values in Panels A and B. (After Vafopoulou and Steel, 2001).

PER/TIM cells in the brain from the PTTH cell except for these dorsal neurons fails to abolish rhythmicity in PTTH release (Nseiri and Steel, 2002), indicating that the dorsal neurons of *Rhodnius* comprise the clock that regulates rhythmic release of PTTH. In pupae of *Antheraea*, PER/TIM cells also lie adjacent to the PTTH cell (Sauman and Reppert, 1996a), where they are thought to regulate the release of PTTH that causes the photoperiodic termination of pupal diapause. The haemolymph PTTH titre of *Bombyx* shows a daily rhythm throughout pupal-adult development (Mizoguchi et al., 2001), raising the possibility that similar, or even the same, neuronal clock machinery may be involved in both circadian and photoperiodic regulation of PTTH release. An anatomically comparable system is found in *Drosophila*. The principle locus of circadian clock neurons in the brain of larval *D. melanogaster* (the lateral neurons) also contain pigment dispersing factor (PDF) (Helfrich-Förster, 1997). The terminal arborisations of these neurons occur in the lateral protocerebrum, and overlap extensively with arborisations of a pair of neurons that directly innervates the PG cells of the ring gland

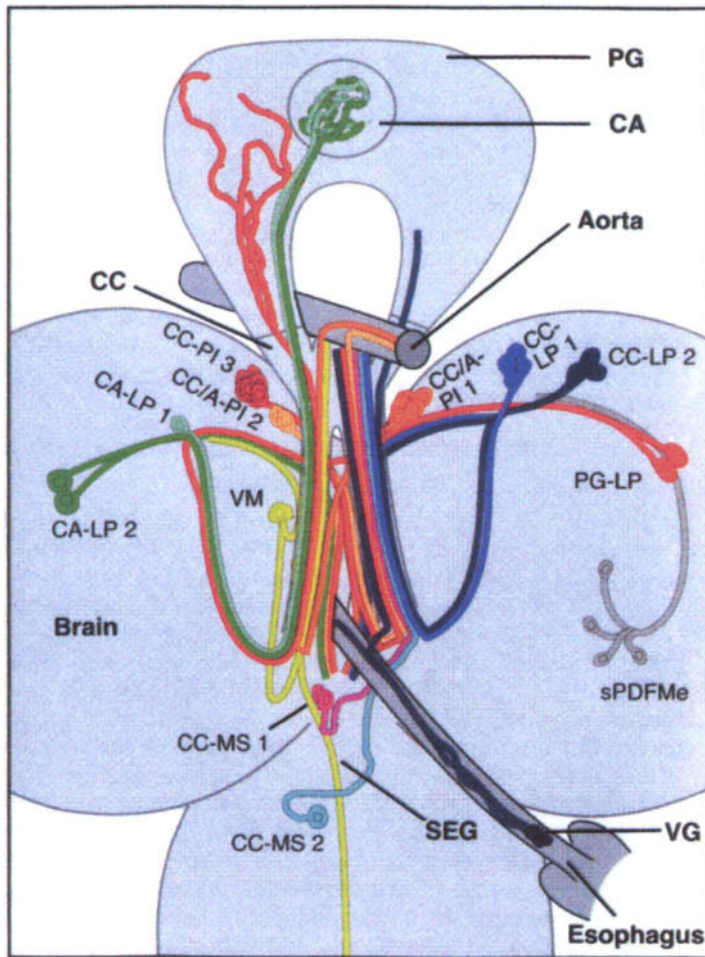


Fig. 5.6. Identified groups of neurons in the brain of *Drosophila melanogaster* and their axonal projections. The ring gland occupies the upper part of the Figure and is subdivided into corpus cardiacum (CC), corpus allatum (CA) and prothoracic gland (PG). SEG is subesophageal ganglion. sPDFMe are the lateral 'clock neurons' that contain pigment dispersing factor. Dendritic fields of these neurons overlap extensively with those of the two PG-LP neurons, which directly innervate the PG and may be the PTTH neurons. The CA is innervated by only three neurons, arranged in two groups (CA-LP1 and 2) one of which passes close to the termination of the sPDFMe neurons. VM neuron is immunoreactive for eclosion hormone and may also interact with the sPDFMe neurons. Five groups of cells project to the CC (CC-LP1 and 2, CC/A-P1, 2 and 3). (From Sigmund and Korge, 2001).

(Sigmund and Korge, 2001) (Fig. 5.6). This pair of neurons resembles the PTTH neurons of moths in location and morphology. Thus, the neuronal machinery for clock control of PTTH is probably present in *Drosophila*. It differs from both moths and *Rhodnius* in that the presumed PTTH neurons make synaptic contacts with PG cells, suggesting PTTH may function as a transmitter or modulator in *Drosophila* rather than as a hormone. A feature common to

Rhodnius, *Drosophila* and moths is the close proximity of 'clock cells' to the PTTH cells. The existence of a group of 'clock cells' regulating PTTH release in the brains of both hemimetabolous and homometabolous insects implies great conservation of clock control over PTTH release and indicates that circadian control is a general and important feature of the regulation of this neurohormone.

A function of PTTH other than that embodied in the classical scheme (i.e. activation of the PGs) is evident from the continuation of rhythmic release throughout development. Indeed, the amplitude of the rhythm is much higher after the HCP (day 6, Fig. 5.3) than before it. By definition, release after the HCP is not required for secretion of new cuticle, nor maintenance of steroidogenesis by the PGs. Thus the greatest quantities of PTTH are released during a period when the classical expectation was that there would be no release at all. The circadian rhythm of PTTH release appears to have a central function in the circadian organisation of larval development and perhaps in adults too (see Section D below).

(b) Moulting hormones

Insect moulting hormones are a family of steroids related to ecdysone and known as *ecdysteroids* (Koolman, 1989; 1990). Over 100 species of ecdysteroid molecules are known. The primary, usually the only, site of ecdysteroid synthesis in larvae is the paired prothoracic glands (PGs). These synthesise *ecdysone* (E) or an immediate precursor which is converted rapidly to E before entering the general circulation. E is converted to *20-hydroxyecdysone* (20E) by the fat body (Grieneisen, 1994). 20E is the main hormonally active ecdysteroid. Ecdysteroids regulate gene expression in target cells; ecdysone-responsive genes possess an ecdysone response element in the promotor region. Ecdysteroids circulate freely in the haemolymph and readily penetrate cell membranes. Therefore, all cells are exposed to titres of ecdysteroids similar to those in the haemolymph (see Steel and Vafopoulou, 1989). Almost all cells of an insect undergo change during moulting in response to ecdysteroids; thus, virtually all cells are both exposed to and respond to, circulating ecdysteroids. In all of many species examined, the haemolymph ecdysteroid titre rises steadily to a peak in the middle of a moult cycle and then declines again to low values at the time of ecdysis (see Steel and Vafopoulou, 1989). The expression of particular genes at specific developmental times is understood as a consequence of these variations of ecdysteroid concentration with developmental time.

Entirely analogous mechanisms appear to operate on the circadian time-scale. Systematic variations in ecdysteroids within a 24 hour period can orchestrate gene expression within a circadian cycle. Moreover, the fact that diverse cells and tissues throughout the insect would receive the same temporal pattern of ecdysteroid signals would enable ecdysteroids to synchronise circadian patterns of gene expression in otherwise unconnected cells and tissues. This capacity of ecdysteroids to function as an 'internal *Zeitgeber*' has resulted in numerous studies that examine the occurrence and clock regulation of circadian changes in ecdysteroids.

The first work that implicated the PGs and ecdysteroids in circadian phenomena was an ingenious but indirect series of experiments in the silkworm, *Samia cynthia*, in which no hormones were actually measured (Mizoguchi and Ishizaki, 1982; 1984a, b). The phenomenon studied was 'gut-purge', which occurs at the end of the feeding period in the fifth instar larva. Gut-purge is gated in *Samia* and some evidence links this behaviour to a small, transient rise in the haemolymph ecdysteroid titre (Fujishita et al., 1982). Thus, the timing of gut-purge was employed as an indicator of ecdysteroid secretion. Larvae from DD were inserted into holes in light-tight partitions with the partition located at different points along the body in each

experiment. The timing of gut-purge was monitored in these animals after a 15 min light pulse was administered on one side of the partition. It was found that a light pulse to the head alone did not affect the timing of gut-purge; if the pulse reached a broad area of the thorax in addition to the head, then a phase delay in gut-purge resulted. In later experiments, three pairs of PGs were implanted into the abdomens of intact animals and a 10 second light pulse was administered to either the front end (containing intrinsic brain and PGs) or to the whole animal (including the implants). The timing of gut-purge was found to be determined by the implanted PGs. The authors concluded that the PGs contained a photosensitive clock that regulates the gating of gut-purge. These fascinating experiments are difficult to interpret for a number of reasons, notably: 1) both the implanted and intrinsic PGs would be subjected to PTTH released (rhythmically?) from the brain, and 2) ecdysteroids from both sets of PGs would interact both with each other and with the brain in ways that cannot be predicted in these experiments (see Steel and Davey, 1985). It was later shown that the small ecdysteroid peak in the haemolymph that normally precedes gut-purge could be shifted in decapitated larvae (Mizoguchi and Ishizaki, 1984a), strengthening the belief that the brain was not required for the above results. Gut-purge itself was not monitored in these later experiments because it was severely disrupted by decapitation (Fujishita and Ishizaki, 1982). A phase-response curve (PRC; see Chapters 2 and 3) of the gut purge rhythm to 15min light pulses in DD was constructed using intact animals (Mizoguchi and Ishizaki, 1984b). The clock governing gut-purge in intact animals yielded a Type 0 PRC (Fig. 5.7), stopped in LL and restarted on transfer to DD. If the belief that gut-purge is controlled by ecdysteroids is correct, the more recent findings of circadian regulation of PTTH release (section C1a above) suggest that this PRC may reflect the integrated functioning of a brain-centred PTTH oscillator and a PG oscillator (see Section C1c below).

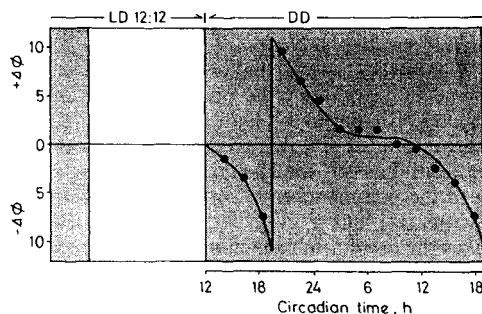


Fig. 5.7. Phase response curve of the gut purge rhythm of the saturniid moth *Samia cynthia ricini*. For discussion, see text. (From Mizoguchi and Ishizaki, 1984a).

Circadian control of ecdysteroids has been dissected by direct measurement in *Rhodnius*. The haemolymph ecdysteroid titre (measured by radioimmunoassay, RIA) is strongly rhythmic, with peaks in each scotophase at 3 to 5 times the values found in adjacent photophases (Steel and Ampleford, 1984). The daily increase in the haemolymph titre anticipates lights-off and free-runs in DD with a period length close to 24 hours which is temperature-compensated (Steel and Ampleford, 1984; Ampleford and Steel, 1985). The rhythm is seen for many consecutive days throughout larval-adult development. It was argued that production of the haemolymph rhythm required both rhythmic synthesis of ecdysteroids (by the PGs) and rhythmic ecdysteroid excretion. The daily drop in haemolymph ecdysteroids

from its peak in the scotophase to its trough in the following photophase was equal to the amount of ecdysteroids found in the faeces each day. It was inferred that the downward side of each daily peak in the haemolymph titre was achieved by removal of ecdysteroids from the haemolymph and their excretion from the body by Malpighian tubules, and possibly the midgut (Steel and Ampleford, 1984). A circadian rhythm of haemolymph ecdysteroids is also seen in larvae of the wax moth *Galleria mellonella*, following exposure to a temperature increase (Cymborowski et al., 1989; 1991). The haemolymph ecdysteroid titre of larvae of *Manduca sexta* also changes in a series of daily 'steps' (Schwartz and Truman, 1983). In *Bombyx mori*, the ecdysteroid titre shows daily increases and decreases in both larvae and pupae (Sakurai et al., 1998; Mizoguchi et al., 2001). A daily rhythm in the ecdysteroid titre is also seen in the cockroach *Periplaneta americana* (Richter, 2001).

Subsequent studies focused on the control of the daily increase in haemolymph ecdysteroid titre by timed synthesis of ecdysteroids by the PGs. Ecdysteroids are small molecules that are not stored in cells and consequently the rate of release of ecdysteroids into the haemolymph is equal to their rate of their synthesis (Vafopoulou and Steel, 1989). A series of experiments was conducted to examine ecdysteroid synthesis by PGs on a circadian time-scale under various lighting regimes. PGs were removed from animals every four hours and the level of ecdysteroid synthesis measured as the amount synthesised during a 4 hour incubation *in vitro* (Vafopoulou and Steel, 1989). Haemolymph ecdysteroid titres were also measured in the animals from which PGs were removed, enabling correlation between changes in levels of synthesis by PGs and the haemolymph titres of the same animals. Synthesis of ecdysteroids by PGs was much higher during the scotophase than the photophase of each day (Vafopoulou and Steel, 1989). The rhythm of synthesis by PGs was found to free-run in DD with a temperature-compensated period (Vafopoulou and Steel, 1991); the daily peaks of synthesis were synchronised with the daily peaks in the haemolymph ecdysteroid titre. Rhythmic ecdysteroid synthesis by PGs has also been shown in larvae of *Galleria* (Cymborowski et al., 1991), *Bombyx* (Sakurai et al., 1998) and *Periplaneta* (Richter, 2001). The complexity of the clock underlying this rhythm in *Rhodnius* began to emerge when animals were transferred from LD 12:12 to LL (Vafopoulou and Steel, 1991); rhythmicity in synthesis was abolished during the first circadian cycle in LL, but re-emerged in the second cycle with peaks in each subjective photophase, i.e. in *antiphase* to its position in DD and LD 12:12. The period length in LL was also shortened. The haemolymph ecdysteroid titre showed the same phase reversal following a lag of one cycle. The results suggested that two circadian oscillators were involved in timing of rhythmic ecdysteroid synthesis *in vivo* (Vafopoulou and Steel, 1991). In this experimental design, some of the observed effects could have been mediated by PTTH released rhythmically from the brain, which acted on the PGs prior to their removal from the animal. Entirely *in vitro* designs were then adopted to dissect the two oscillators from one another.

One oscillator was traced to the PG cells themselves using entirely *in vitro* experiments, in which any influence of rhythmic PTTH is excluded. PGs incubated *in vitro* were exposed to transfer from LL to DD or vice-versa during incubation (Vafopoulou and Steel, 1992). Ecdysteroid synthesis underwent abrupt acceleration following a 'lights-off' signal, but was unaffected by 'lights-on'. Thus, PGs are directly photosensitive *in vitro* and respond to light signals. That these responses were generated by a photosensitive circadian oscillator in the PGs was demonstrated by Vafopoulou and Steel (1998). In these experiments, PGs were rendered arrhythmic by prolonged exposure to LL in the intact animal and then incubated *in vitro* for 42 hours. A 'lights-off' signal given to arrhythmic PGs *in vitro* induced the abrupt acceleration of synthesis seen in 1992, followed by a free-running rhythm of synthesis (Fig. 5.8). Thus, rhythmicity is induced by 'lights-off'. These experiments demonstrated that PGs contain a

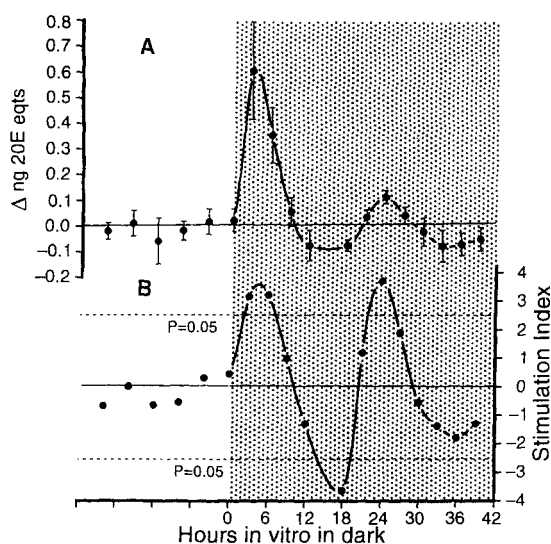


Fig. 5.8. Induction *in vitro* of a free-running rhythm of ecdysteroid synthesis in prothoracic glands of fifth instar larvae of *Rhodnius prolixus* following transfer of glands from LL (clear area) to DD (stippled area). Panel B depicts the changes in statistical significance of the numerical values in Panel A. (From Vafopoulou and Steel, 1998).

photosensitive circadian oscillator that drives ecdysteroid synthesis. In turn, ecdysteroid synthesis by PGs drives the upward side of the haemolymph titre rhythm each day. These findings confirmed and extended the concept of a 'PG clock' invoked initially by Mizoguchi and Ishizaki (1982) in *Samia*. In prepupae of *Drosophila*, the *period* gene is expressed rhythmically in the region of the ring gland that corresponds to the PGs (Emery et al., 1997). Using cultures of ring glands attached to the central nervous system (CNS) *in vitro*, it was found that this rhythm of *per* expression was light-entrainable. The rhythm persisted in the presence of a concentration of tetrodotoxin (TTX) in the medium that was sufficient to block nervous conduction, showing that cycling *per* expression was not driven by the attached CNS. A 'PG clock' is therefore also present in *D. melanogaster*. However, there is no evidence of a rhythmic output from the cells; the numerous reports of ecdysteroid titres in *Drosophila* (see Richards, 1981) revealed no evidence of rhythmicity. A 'PG clock' was also invoked by Cymborowski et al. (1991), who found that the rhythm of haemolymph ecdysteroids in *Galleria mellonella* was not abolished by decapitation. Conversely, Richter (2001) found that neck ligation of *Periplaneta* larvae caused depression of ecdysteroid synthesis by PGs to a level where the rhythm seen in intact animals was undetectable. It is not yet known whether the ecdysteroid rhythms reported in *Bombyx* (Sakurai et al., 1998; Mizoguchi et al., 2001) are driven by the PTH rhythm seen in this species or by the 'PG clock' of earlier studies (Mizoguchi and Ishizaki, 1982).

Is this 'PG clock' localised within the glands? Each PG consists of about 200 structurally identical gland cells. In *Rhodnius* these cells receive no nerve supply (Wigglesworth, 1952) by which the cells might be synchronised. By contrast, lepidopteran PGs

are richly innervated (Lee, 1948). Each cell appears capable of ecdysteroid synthesis (Asahina et al., 1994). The cells are interconnected by gap junctions (Dai et al., 1994) and calcium-dependent action potentials (Eusebio and Moody, 1986), which presumably synchronise the population of cells into a coherent whole. Every cell of the PG is immunoreactive for the clock proteins PER and TIM (Terry and Steel, 2002); these proteins exhibit the cycling and nuclear migration characteristic of cells that generate circadian oscillations. The cycling of PER in the PG cells of *Rhodnius* is illustrated in Figs 5.10a and 5.10b. Thus, every PG cell appears to be a 'clock cell' (Chapter 4). PG cells are one of rather few groups of 'clock cells' for which both the entrainment and output pathways have been characterised (see Section A2). The gland behaves as a coherent, rhythmic whole because of coupling pathways between individual PG cells. This construction of clocks from cellular oscillators coupled by gap junctions and local potentials has been described for neuronal clocks in the eye of the mollusc, *Bulla* (Block et al., 1993) and the mammalian suprachiasmatic nucleus. Thus, the 'PG clock', though an endocrine gland, seems remarkably similar to neuronal clocks. It is of interest that PGs are, like the nervous system, of ectodermal origin (Lee, 1948).

The second circadian oscillator that was found to influence rhythmic ecdysteroid synthesis by PGs in *Rhodnius* is the oscillator in the brain that regulates the rhythm of PTTH release. The PGs are the only known target of PTTH, so an effect of PTTH on the PGs is expected. However, the above experiments showing that the 'PG clock' operates entirely *in vitro* exclude the possibility that the rhythm in PTTH directly *drives* the rhythm of steroidogenesis in the PGs. It is worth noting that in most endocrine systems, it is assumed that neuropeptide hormones (e.g. from the pituitary) directly drive rhythms in steroid synthesis in endocrine glands. It has been suggested that the assumption that steroid endocrine rhythms are slaves to neuronal pacemakers may benefit from re-evaluation (Vafopoulou and Steel, 1998). That PTTH was not needed for rhythmic steroidogenesis was demonstrated *in vivo* by Pelc and Steel (1997). *Rhodnius* larvae were either decapitated or injected with TTX (a blocker of voltage-dependent sodium channels) at a dose shown to cause flaccid paralysis of the whole animal and to prevent release of PTTH for four days. Rhythmic steroidogenesis by PGs was maintained in both cases; the rhythm of both synthesis and haemolymph titre retained entrainment to a light-dark cycle and free-ran in both DD and LL. In both paralysed and decapitated animals, the rhythms of both steroidogenesis and titre showed a reversal of phase from that of intact animals under all conditions of illumination. Collectively, these data showed that when PTTH is eliminated, the rhythm of ecdysteroid synthesis runs in antiphase to its position when PTTH is present. It was concluded that when PTTH is absent, the PGs entrain directly to the light cycle and adopt a day-time peak. But in the presence of a rhythm of PTTH, the PGs adopt a night-time phase. Therefore, the rhythm of PTTH acts as an entraining agent to the 'PG clock'. It may be significant that PTTH is known to act on the PGs by regulating the influx of calcium into the cells (see Smith 1993), since calcium is known to influence circadian phase in many systems. Pulses of calcium generate PRCs which are usually displaced by 12 hours from those generated using light pulses (Edmunds, 1988). This calcium transduction pathway is therefore an appropriate one by which PTTH could convey phase control information to the cellular oscillators of PG gland cells. When both light and PTTH are present, as in intact animals, PGs entrain preferentially to PTTH. It is notable that both PTTH and light can act as a *Zeitgeber* to the 'PG clock', but they contradict rather than reinforce each other.

It is apparent from the above that both PTTH and ecdysteroids are regulated by circadian oscillators, and these two endocrine rhythms interact every day. This picture is quite different from classical expectations (e.g. Steel and Davey, 1985), in which hormones were envisaged as acting sequentially rather than 'in tango'. The nature of the daily interactions between the rhythms of PTTH and ecdysteroids was elucidated in a series of experiments *in vivo* (Vafopoulou and Steel, 2001).

(c) Multioscillator organisation of developmental hormones

The two preceding sections show that multiple photosensitive circadian clocks are operating simultaneously during development in *Rhodnius* and probably in other insects. A group of clock neurons is located adjacent to the PTTH cells in the dorsal protocerebrum and regulates the rhythmic release of PTTH. This machinery is bilaterally duplicated in the two brain hemispheres. Thus, there are two PTTH clocks in the brain. These are presumably synchronised by nervous connections between left and right hemispheres, as with optic lobe clocks (see Chapter 8). In addition, each PG is a photosensitive circadian clock. Left and right PGs are coupled *in vivo*, since both PGs are synchronised with the ecdysteroid levels in the haemolymph. Therefore, a total of four clock loci participate in regulation of the hormones controlling development in *Rhodnius*. Four similar loci are less fully documented in moths, where information is distributed between the genera *Antheraea*, *Bombyx*, and *Samia*. Circadian clock neurons are closely associated with PTTH cells in the brain in three divergent species, *Antheraea pernyi*, *Drosophila melanogaster* and *Rhodnius prolixus*. In *Drosophila*, the number of oscillators is reduced to three due to incorporation of the two PGs into a single ring gland. In *Rhodnius*, each of the four loci comprises a population of cellular oscillators that show cycling of 'clock' proteins, thus each clock locus is a multioscillator unit (see also Chapter 6). The coordination mechanisms within this three- or four-oscillator system have been examined *in vivo* in *Rhodnius* (Vafopoulou and Steel, 2001). In animals maintained in LL, the rhythm of PTTH release damps after five cycles and then release ceases completely. When such animals were transferred to DD, a free-running rhythm of PTTH release was initiated with peaks in each subjective photophase (Fig. 5.5). Rhythmic ecdysteroid synthesis was also initiated in the PGs of these animals, but the first peak of ecdysteroid synthesis occurred within 7 hours of transfer to DD, many hours *before* the first peak of PTTH release in the same animals (Fig. 5.9), showing that rhythmicity in steroidogenesis is not *driven* by rhythmicity in PTTH. This conclusion is confirmed by the finding that the time of this first peak in ecdysteroid synthesis was unaffected when PTTH release was prevented by prior injection of TTX and was similar to that seen in PGs that were exposed to the same light cue entirely *in vitro* (Fig. 5.8). This prompt response of PGs *in vivo* is therefore due to a direct response of the PGs to the light cue, not to PTTH. Therefore, the 'PG clock' is photosensitive *in vivo*, despite the thick layer of brown cuticle that lies over it. Therefore, both the brain 'PTTH clock' and the 'PG clock' are operational in the intact animal. However, the subsequent behaviour of rhythmic steroidogenesis in TTX-injected animals was different from that of normal animals, revealing the influence of PTTH on the PGs. In TTX paralysed animals, ecdysteroid synthesis dropped rapidly during the first subjective photophase. By contrast, the first peak in normal animals is much broader and high levels of synthesis are prolonged into the first subjective photophase (Fig. 5.9). It is during this first subjective photophase that the first peak of PTTH release occurs. Throughout subsequent cycles, steroidogenesis in normal animals continues to peak during the subjective photophase in synchrony with the peaks of PTTH release. But in the

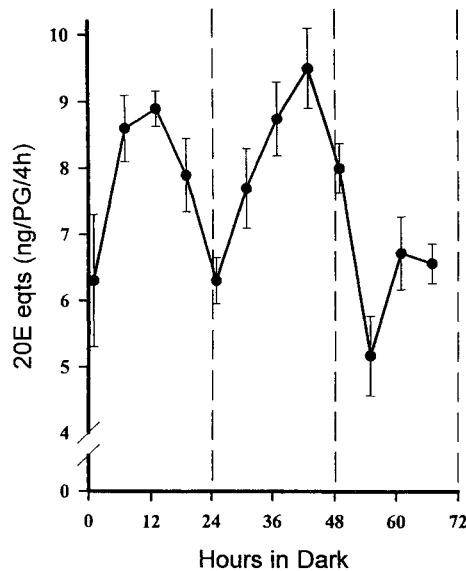


Fig. 5.9. Induction *in vivo* of a free-running rhythm of ecdysteroid synthesis in prothoracic glands of fifth instar larvae of *Rhodnius prolixus* by transfer of larvae from LL to DD. (From Vafopoulou and Steel, 2001).

absence of PTTH (TTX-paralysed animals), the first sharp peak in steroidogenesis recurs each subjective scotophase. Therefore, the first induced release of PTTH in normal animals shifts the phase of the rhythm of steroidogenesis from subjective night to day. These findings, and those of Pelc and Steel (1997), show that rhythmic release of PTTH regulates the *phase* of rhythmic steroidogenesis in the PGs. Thus, expression of the 'PG clock' can be induced by light, but the induced oscillations are then entrained by PTTH. PTTH seems to dominate light *in vivo* as a *Zeitgeber* to the 'PG clock'.

PTTH seems to act as a hormonal *Zeitgeber* to the PGs, which coordinates the 'PTTH clock' and the 'PG clock' together into a functional unity. The PG cells do detect the daily signals from PTTH; they have a rhythm of responsiveness to PTTH (Vafopoulou and Steel, 1999) that peaks at the natural time of PTTH release each day. This rhythm of responsiveness probably results from a daily cycling of PTTH receptor availability in the PG cells (Vafopoulou and Steel, 1999), but whether it is driven by PTTH or the 'PG clock' is not yet known. Since the PGs of *Rhodnius* receive no nerve supply (Wigglesworth, 1952), the PTTH rhythm presumably also synchronises left and right PG clocks. There is a large literature on feedback effects of ecdysteroids on PTTH release from the brain (Steel and Davey, 1985), so reciprocal action of the ecdysteroid rhythm on PTTH release can also be anticipated. Indeed, ecdysteroid receptors (EcR) are seen by immunohistochemistry in many neurosecretory cells of the dorsal protocerebrum (Fig. 5.10c, 5.10d). EcR cycles in these cells, indicating the neurosecretory cells respond rhythmically to ecdysteroids. Of special interest is the fact that EcR in these locations does not cycle in synchrony with the haemolymph ecdysteroid titre, showing that EcR is not induced by ecdysteroids. Rather, the neurosecretory cells themselves seem to determine the

time of day when they will be sensitive to ecdysteroids. This behaviour is different from that seen in other target tissues (see below).

Another hormone that may participate in this regulatory system is melatonin. Melatonin is synthesised by several insect tissues and has been found to undergo variations within a day in some cases (Linn et al., 1995). At least in *Rhodnius*, melatonin levels in the haemolymph are rhythmic and under circadian control (Gorbet and Steel, 2002). It has been found that exogenous melatonin promotes the release of PTTH from brains of *Periplaneta* *in vitro* (Richter et al., 2000). These findings suggest that interactions between the rhythms of melatonin and PTTH may be a further part of this multioscillator system. Such a role for melatonin may be anticipated from its function in vertebrates.

The neuroendocrine axis that regulates development is therefore seen, at least in *Rhodnius*, to comprise four anatomically distinct loci of photosensitive circadian oscillators that are coordinated into a functional multioscillator system by nerves and hormones. Both hormones are released every day under the control of their respective clocks, but are coordinated by daily regulatory interactions. These hormonal interactions have been characterised as a daily 'tango' between PTTH and ecdysteroids, rather than the sequential 'cascade' described in the older literature (Steel and Vafopoulou, 1999; 2001).

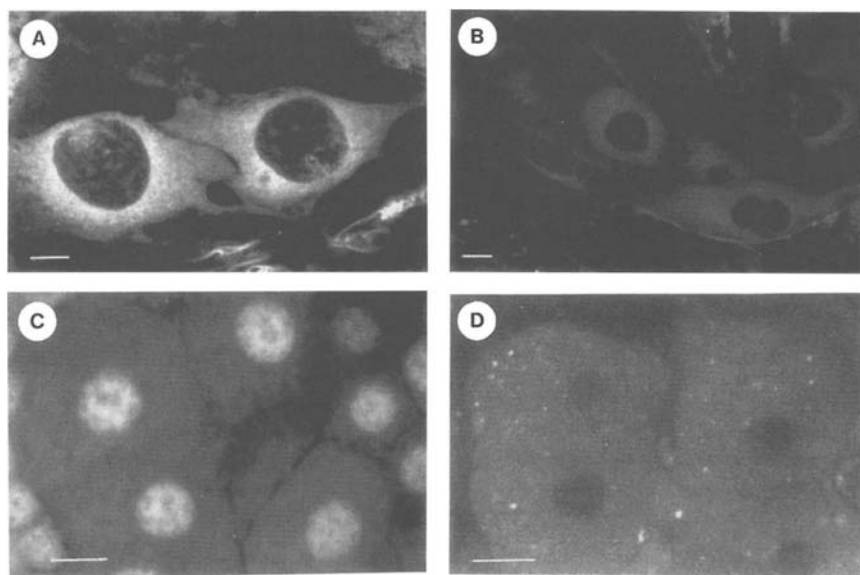


Fig. 5.10. Confocal microscope images showing immunoreactivity of prothoracic gland cells to PER (A, B) and brain protocerebral neurosecretory cells to ecdysteroid receptor (EcR) (C, D) in the middle of larval-adult moult of *Rhodnius prolixus*. Prothoracic gland cells show strong, abundant immunobinding in both nucleus and perinuclear cytoplasm during the scotophase (A), and no binding during the photophase (B). The neurosecretory cells in the brain that synthesise bombyxin show intense nuclear immunofluorescence during the beginning of the scotophase (C), but none during the beginning of the photophase (D). Scale bars = 10 μm . A and B are kind gifts from K.L. Terry. See text for details.

The complexity of this timing system attests to a requirement in the insect for very precise temporal control over the output. This output of the timing system is the circadian rhythm in haemolymph ecdysteroids. Ecdysteroids penetrate all tissues readily, indicating that all cells will be exposed to rhythmic changes in ecdysteroid levels similar to those occurring in the haemolymph. Further, almost all cells respond to ecdysteroids, thus the whole insect experiences rhythmic changes in ecdysteroids. Receptors for ecdysteroids are found in almost all tissues and in several *Rhodnius* tissues these receptors show daily cycling (Vafopoulou et al., 2001). Therefore, the haemolymph ecdysteroid titre rhythm is detected by target tissues, which then respond rhythmically. Most cells possess ecdysone-responsive genes and these are known to be expressed in temporally orchestrated sequences (Cherbas and Cherbas, 1996). Thus, the haemolymph ecdysteroid titre rhythm provides temporal information to target cells with which they can orchestrate sequential gene expression during a circadian cycle (Steel and Vafopoulou, 1999; Vafopoulou et al., 2001). EcR cycles in traditional target tissues such as epidermal cells (Vafopoulou et al., 2001), showing that they are sensitive to the temporal information in the ecdysteroid titre. The EcR reactivity in epidermal cells resembles that in the neurosecretory cells (Fig. 5.10c and 5.10d) with the important difference that the cycling in epidermal cells is in synchrony with the ecdysteroid titre. This synchrony suggests that epidermal EcR is induced by ecdysteroids, which implies that epidermal cell responses are driven by the ecdysteroid titre. In larvae of the midge *Chironomus tentans*, region I-18C of the salivary gland chromosomes exhibits a daily rhythm of condensation and decondensation (Lezzi et al., 1991); decondensation (associated with transcriptional activity) during the scotophase parallels increased responsiveness to exogenous 20E. Most such target cells lack mechanisms with which to measure time or to synchronise themselves with the environment. The ecdysteroid rhythm appears to distribute temporal information throughout the insect and provide time cues to developing cells. Since all the cells of numerous tissues are exposed to the same hormonal time cues each day, the various cells and tissues will become synchronised both with each other and with the external world. The multioscillator timing system in the PTTH-ecdysteroid axis seems to be a central mechanism by which temporal order is generated and maintained during development. This regulatory scheme is summarised in Fig. 5.11. It should be noted that these hormones continue to be present in adult insects (Section D1). Their persistence throughout the life cycle supports the possibility that they may comprise a central timing system for the insect (see also Section A3).

2. Ecdysis

Ecdysis is the term for the complex of behaviours by which an insect extracts itself (emerges) from the confines of an old exoskeleton. The term eclosion refers exclusively to the adult ecdysis of holometabolous insects. The subject of ecdysis, and eclosion in particular, has long been of special interest to circadian biologists. The conceptual foundations of modern circadian biology were established by Pittendrigh and his associates through elegant analyses of the formal properties of the eclosion rhythm of *Drosophila* (see Chapter 3). The theoretical appeal of using the *Drosophila* eclosion rhythm as a model for physiological analysis is impractical because of the small size of the fly. Modest information has been obtained concerning the location of the photoreceptors and clock by physiological methods and is summarised in Chapters 4 and 8. *Drosophila* continues to be the insect of choice for genetic

manipulations that have led to the identification of 'clock cells' (Chapter 4) and to the construction of eclosion hormone knockouts (see below).

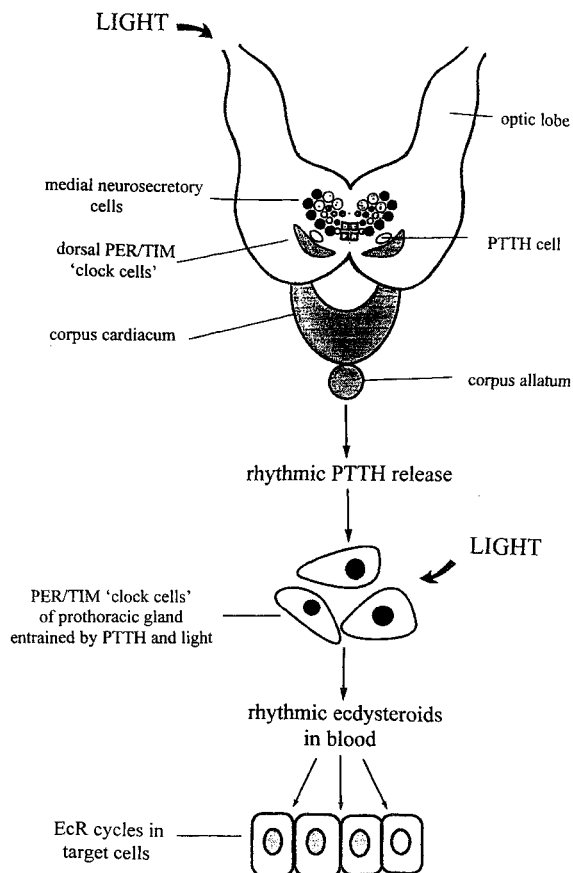


Fig. 5.11. Schematic summary of the multis oscillator timing system that regulates developmental hormones in *Rhodnius prolixus*. A paired group of neurons that exhibit circadian cycling of PER and TIM ('clock cells') is located in the dorsal protocerebrum. These 'clock cells' are entrained by light received through the eyes (but see text) and regulate the rhythmic release of the neuropeptide prothoracicotropic hormone (PTTH). PTTH is made in a single paired PTTH cell close to the 'clock cells'. It is released into the haemolymph from the corpus cardiacum/corpus allatum complex and circulating levels are rhythmic. The prothoracic glands (PGs) also exhibit circadian cycling of PER and TIM and are also entrainable by light. Synthesis of ecdysteroids is rhythmic and controlled by this 'PG oscillator'. *In vivo*, PTTH acts as a hormonal *Zeitgeber* to the 'PG oscillator' and entrains the rhythm of ecdysteroid synthesis preferentially to light. It also couples left and right PGs. This circadian system comprises at least four oscillators (two in the brain, one in each of the two PGs) which are coupled by nerves and hormones (see text). The circadian rhythm of ecdysteroids in the haemolymph is detected by almost all cells in the insect, which show cycling of the ecdysteroid receptor (EcR) in synchrony with the hormone rhythm. Thus, ecdysteroids drive rhythmic gene expression (see text) in target cells, thereby orchestrating gene expression within a circadian cycle.

The complex of behaviours involved in ecdysis is usually divided into at least two stages: a pre-ecdysis sequence in which remaining attachments between old and new integuments are loosened, and an ecdysis sequence dominated by rhythmic peristaltic contractions of the abdomen which drive the body out of the old cuticle. Detailed descriptions for various species are given by Reynolds (1980) and Ewer and Reynolds (2002). Circadian rhythms in ecdysis have been reported in many orders of both exopterygote and endopterygote insects (see Chapter 3).

The underlying control mechanisms have been examined in only a few species (see Ewer and Reynolds, 2002), and detailed information derives almost entirely from the extensive studies on moths, notably the saturniids *Antheraea pernyi* and *Hyalophora cecropia*, the sphingid *Manduca sexta* and the silkworm *Bombyx mori*.

It is obvious that a new exoskeleton must be formed below the old one prior to ecdysis. Thus, ecdysis is a complex of behaviours which is timed in relation to preceding developmental events. Circadian control of these developmental events can potentially lead to consequential circadian control over ecdysis. In some situations, there may be, in addition, direct circadian control over the timing of onset of ecdysis behaviour. Therefore, the timing of ecdysis may be the result of the combined operation of two sets of circadian systems. The first of these systems has been described above (Section C1); this section addresses the second system and its mechanism of coordination with the first.

Some introductory comments concerning the nature of ecdysis and the significance of temporal control of it are needed. During a cycle of moulting, the epidermal cells detach from the exoskeleton (*apolysis*) and then secrete a new exoskeleton beneath the old one. Apolysis occurs at the peak of ecdysteroid titres in each instar, which is roughly half way between the initiation of the moulting process and ecdysis (see Steel and Davey, 1985; Steel and Vafopoulou, 1989). In the present context, the important feature is that from apolysis onwards, the insect is surrounded by two exoskeletons, the outer of which hangs loosely about the insect like an ill-fitting suit of armour. This loose outer exoskeleton poses various problems for the insect. Feeding becomes increasingly difficult, particularly where chewing with loose mandibles is required. Locomotion is impaired as if the legs were encased in waders. Perhaps the most serious problem is impaired gas exchange. The tracheal system is epidermal and lined throughout its entire length with cuticle. The entire cuticle lining of the tracheal system is extracted through the spiracles and discarded at ecdysis. From apolysis onwards in a moult cycle, all the trachea possess two cuticular linings, the old lining being loose within the new one. The flow of air through the tracheal system is partially obstructed by the old lining, and therefore the efficiency of gas exchange is reduced. These impairments to feeding, locomotion, breathing and doubtless other processes, render insects vulnerable to predators during this time, leading them to take shelter in secluded locations. The point of this discourse is to stress that a strong selective pressure exists for ecdysis to take place as early as possible in the moulting process. As soon as the new cuticle is thick enough to provide adequate structural support (to internal tissues and the insect as a whole), the old cuticle is shed at ecdysis, inflated (by swallowing air or water) and then hardened by quinone tanning of cuticular proteins. At the time of ecdysis, the new cuticle is generally only half (or less) its final thickness; cuticle secretion continues for days or even weeks after ecdysis (see Section C3). Numerous other physiological changes (e.g. in neural circuitry and muscle development) also continue. It is a widespread error in the literature to refer to ecdysis as occurring at the end of the moult, or to

state that it is the final event in moulting. In fact, ecdysis occurs about half way through the moulting process.

In light of the above, ecdysis is seen as a set of behaviours that are activated about half way through a moult cycle. The physiological requirement for coordination of ecdysis with the progress of moulting is therefore self-evident and presumably universal. The significance of the second level of circadian control, the direct control of timing of the behaviours, is less obvious. Some insects display a direct circadian control of ecdysis, while others do not (see Reynolds, 1980). An early speculation was that *Drosophila* emerged around dawn because the humidity was high at that time. However, in many species emergence is confined to the photophase, when humidity is at its daily minimum. Possibly, circadian control of the adult ecdysis is an adaptation that ensures the synchronous emergence of a population of adults that will commence reproduction together. Closely related species may have eclosion gates that are separated by many hours. Such species differences in eclosion time may represent a mechanism that promotes reproductive isolation. Thus, gated ecdysis of adults may serve to synchronise subsequent reproductive activities. The progress of development determines which of the recurrent daily gates will be used by an individual insect. These considerations imply that gated adult ecdysis would be critical to species in which the adults mate soon after emergence, particularly if they lay only one batch of eggs and then die. This scenario is commonly seen in the Lepidoptera. The expectation of precise temporal control over emergence, combined with their large size, has resulted in intensive analysis of the control over eclosion in moths, commencing with the classic studies of Truman and Riddiford (1970).

The subject of the control of ecdysis contrasts with many other sections in this chapter in two general ways. First, the literature dealing with physiological mechanisms underlying eclosion is vast; the review by Ewer and Reynolds (2002) contains almost 300 references. Second, while most sections of this chapter have never been reviewed previously, the subject of ecdysis has been reviewed frequently and thoroughly; about two dozen reviews have been published since 1978 and new reviews have appeared annually since 1990. Therefore, it is both inappropriate and impossible to review the subject of ecdysis as comprehensively as other topics in this chapter. Some of the most informative and comprehensive reviews are those of Reynolds (1980), Truman (1985; 1992), Horodyski (1996) and Ewer and Reynolds (2002). Shorter reviews and reviews of specific aspects of the literature are those of Truman (1978a; 1990), Reynolds (1987), Truman and Morton (1990), Truman et al. (1991), Hesterlee and Morton (1996), Ewer et al. (1997), Predel and Eckert (2000), Nässel (2000), Zitnan and Adams (2000) and Jackson et al. (2001). The last five articles listed all give explicit schematic models of the pathways of nervous and hormonal interactions that underlie eclosion, as seen from their authors' respective viewpoints.

The early studies of eclosion (see Chapters 3 and 8) were conducted using saturniid moths, primarily *Antheraea pernyi* and *Hyalophora cecropia*. Eclosion is a gated event in a population and free runs in aperiodic conditions, hence is under circadian control. Gated eclosion was retained following removal of the eyes, suboesophageal ganglia or corpora cardiaca (Truman and Riddiford, 1970). Removal of the brain resulted in eclosion that was arrhythmic. Brain transplantation experiments demonstrated that both photoreceptor and clock were located in the brain and the output of the clock was a humoral factor. This factor became known as eclosion hormone (EH). Details of these experiments are given in Chapter 8. It is important to note that removal of the brain eliminates the *timing* of eclosion but does not prevent eclosion from taking place (Truman and Riddiford, 1970; Truman, 1972). When

'loose-brains' of *Antheraea pernyi* were implanted into brainless *Hyalophora cercopia* (or vice-versa), the *phase* of the eclosion gate was determined by the implanted brain (see Fig. 8.7), but the eclosion *behaviour* movements were those of host. Eclosion hormone is considered a gating hormone whose release regulates the timing of eclosion but is not required for eclosion to occur. The mechanism by which eclosion occurs in the absence of EH is still debated (Truman, 1992). However, rather few brainless moths execute eclosion behaviour normally (Truman, 1971) and none subsequently inflate their wings (Truman and Riddiford, 1974b). The eclosion that occurs following implantation of a loose brain resembles that of intact animals much more closely, implying that EH plays a role in coordination of the expression of behavioural routines.

Extracts of corpora cardiaca containing EH were shown to trigger patterns of neural activity in abdominal ganglia that correspond closely to the abdominal movements associated with eclosion. This was shown first *in vivo* in a de-afferented CNS preparation (Truman and Sokolove, 1972) and then in the isolated abdominal CNS *in vitro* with its tracheal supply attached (Truman, 1978b). An exposure to EH of only 5 min was sufficient to trigger the entire motor sequence; thus, the abdominal ganglia contain both pattern generators for particular movements (e.g. wriggling, peristalsis) and for the longer term progression of the behavioural sequence from one phase to the next. These experiments were the basis for the long standing belief that EH acted directly on the CNS to trigger ecdysis behaviour. Later work (Zitnan et al., 1996) attributed these findings to the action of EH on epitracheal glands included in this preparation (see below).

The older literature attributes a variety of other changes that occur at ecdysis to EH. These include activation of the 'gin-trap' reflex in the pupa (Levine and Truman, 1983), rendering the cuticle of the pharate adult wing extensible (Reynolds, 1977; 1985), regulating the release of the hormone *bursicon*, which tans the cuticle after ecdysis (Reynolds et al., 1979) and stimulating the cement glands below the epidermis (Hewes and Truman, 1991). More recent studies have revealed that three additional neuropeptides (PETH, ETH, CCAP) released from other sites in the insect (discussed below), are crucial to the regulation of ecdysis. These findings raise questions about which of these reported effects of EH are direct and which are indirectly mediated by other peptides (see Ewer and Reynolds, 2002).

Irrespective of the detailed mechanisms by which they are achieved, it is clear that ecdysis behaviours are closely integrated with a variety of other physiological changes that occur around the same time. This integrated package of events at ecdysis must be coordinated very precisely so that it occurs at the optimum moment with respect to both the course of development and the time of day. There is extensive evidence that the ecdysis control system obtains information about the course of development primarily from the haemolymph ecdysteroid titre. Consequently, ecdysteroid levels play a central role in timing of ecdysis. It will be recalled that circadian mechanisms govern both ecdysteroids and PTH (Section C1). Therefore, these developmental hormones are potentially able to provide the ecdysis control system with information about time of day as well as developmental time. Evidence of this view is given below.

In the silkworm *Samia cynthia*, all four larval ecdyses (Fujishita and Ishizaki, 1981) and the pupal ecdysis (Fujishita and Ishizaki, 1982) are tightly gated. The ecdysis rhythm free runs in DD, damps in LL and shows temperature compensation. Therefore, ecdysis is under circadian control. In *Manduca sexta*, larval ecdyses are also rhythmic (Truman, 1972). Truman (1972) found that ecdysis of *Manduca* to the fifth instar occurred a relatively fixed length of

time after the head critical period (HCP). Since the HCP is a gated event, it was inferred that the gating of larval ecdysis was consequent upon the gating of prior endocrine events that control moulting. These events were presumed to involve the circadian control of PTTH release and, by association, ecdysteroid levels (see Ewer and Reynolds, 2002, and Section C1). The finding that the levels of ecdysteroids influenced the timing of ecdysis in *Tenebrio* (Slama, 1980) suggested mechanisms whereby information concerning the progress of development might be integrated into mechanisms that time ecdysis in larvae (Truman et al., 1983). Injection of 20E into fifth instar *Manduca* within 8 hours prior to ecdysis resulted in a dose-dependent delay in ecdysis (Truman et al., 1983). Similar injections into pharate adult *Manduca* also induced delays in eclosion, but in this case the delay was discontinuous, with the animals jumping to progressively later eclosion gates as the dosage of 20E increased (Fig. 5.12). This discontinuous response of eclosion to 20E was seen as a manifestation of the direct circadian control over eclosion that is absent at previous ecdyses. Thus, a decline in the titre of ecdysteroids is a prerequisite for ecdysis of all stages.

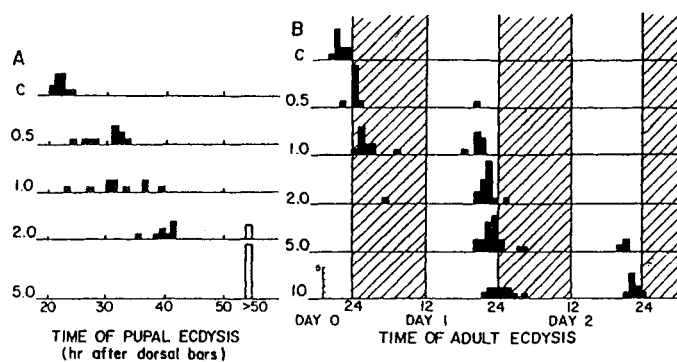


Fig. 5.12. Increasing delays of the time of pupal ecdysis (A) and adult eclosion (B) following injections of increasing amounts of 20-hydroxyecdysone (20E) in *Manduca sexta*. The time of pupal ecdysis is delayed progressively as the dose of 20E is increased. The same is true for eclosion for small doses of 20E, but at larger doses the time of eclosion is displaced into the following gate. Open bars indicate animals which did not attempt ecdysis and C shows the time of ecdysis of control animals (no injection). (From Truman et al., 1983).

This steroid drop provides information about developmental readiness for ecdysis through at least two distinct mechanisms of action. First, ecdysteroids regulate the release of EH; EH release is inhibited until the ecdysteroid levels fall to a critical level. This mechanism assures the coordination of EH release with the progression of development. Second, the steroid drop is required for the induction of sensitivity to EH; 20E injections delay the onset of behavioural responsiveness to EH (Truman et al., 1983; Morton and Truman, 1988). It should be noted that these relationships between ecdysteroids and behaviour are not affected by how or where in the insect the ecdysteroids may act. It is apparent from the above that activation of ecdysis behaviours is very tightly locked into the ecdysteroid titre, both at the level of EH release and of behavioural responsiveness to EH.

In addition to the above, levels of ecdysteroids are also potentially able to provide the system controlling ecdysis with information concerning time of day, and thereby to regulate the

gating of ecdysis. The precision with which ecdysis is gated in various species may therefore be related to the precision with which their ecdysteroid titres are timed. In *Bombyx mori*, ecdysis is tightly gated (Sakurai, 1983) and the ecdysteroid titre exhibits a daily rhythm (Sakurai et al., 1998; Mizoguchi et al., 2001); this rhythm could provide the ecdysis system with temporally precise signals. Conversely, the ecdysteroid titre in *Manduca* declines in a series of relatively imprecise daily steps (Schwartz and Truman, 1983), which could explain the weak gating of larval ecdysis reported by Truman (1972a). In both *Manduca* (Truman, 1972a) and *Bombyx* (Sakurai, 1983), the timing of ecdysis is set early in development; in *Bombyx* the ecdysis time is set on the day that rhythmicity in the circulating ecdysteroids commences (Sakurai et al., 1998). Circadian control of ecdysteroids has been most fully studied in *Rhodnius prolixus* (see Section C1b), and all ecdyses are tightly gated (Ampleford and Steel, 1982). In *Rhodnius*, ecdysis remains phase-shiftable for many days after the onset of circadian rhythmicity in circulating ecdysteroids (Ampleford and Steel, 1982; 1985), apparently because the ecdysteroid rhythm is itself entrainable to light (see Section C1b). The close relationship between the rhythms in ecdysteroid titre and in ecdysis is illustrated by the finding that manipulations of the ecdysteroid titre rhythm lead to predictable changes in the ecdysis rhythm (Ampleford and Steel, 1986). Even so, detailed comparison of the rhythms in ecdysteroids and ecdysis revealed that the ecdysteroid rhythm alone did not directly drive the rhythm in ecdysis and that other factors (such as EH?) were involved (Ampleford and Steel, 1986). Thus, circadian time cues present in the circulating ecdysteroid titre seem to provide signals to the ecdysis control system that regulate the gating of ecdysis.

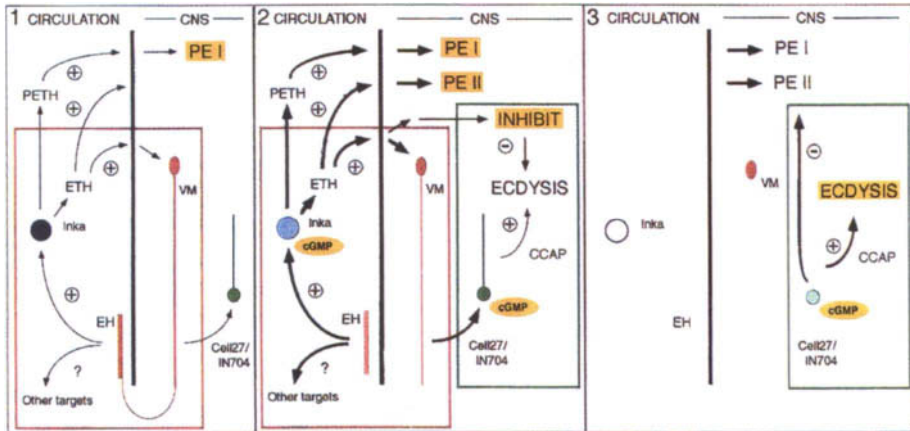
In conclusion, the ecdysis control system reads the levels of circulating ecdysteroids to determine developmental readiness for ecdysis, i.e. which of the daily gates will be used by an individual insect. The ecdysteroid levels contain circadian information in at least some species and this information could be used to time ecdysis within a day, i.e. to establish the gated nature of ecdysis. Indeed, it would be strange if such circadian input to the ecdysis control system was ignored.

EH is synthesised in VM (ventromedial) cells in the tritocerebrum of the brain. In *Manduca*, there are two pairs of VM cells (Truman and Copenhaver, 1989) and in *Drosophila* one pair (Horodyski et al., 1993). In adult *Drosophila*, each cell has three branches (Siegmund and Korge, 2000) (see Fig 5.6). The first branch terminates in the vicinity of terminals of the lateral 'clock neurons' in the protocerebrum, suggesting a potential site of circadian input at eclosion. The second branch terminates in the corpus cardiacum region of the ring gland, a major neurohaemal organ. The first two branches are not seen in the larval stages of *Manduca* (Truman and Copenhaver, 1988; Truman, 1992). The third branch passes posteriorly through the CNS and terminates in an extended neurohaemal site along the proctodeal nerve (Truman and Copenhaver, 1989). EH release is also believed to occur from these axons within the ventral ganglia of the CNS (Hewes and Truman, 1991), where it could act locally within the CNS; antidromic stimulation of these axons after severance of the proctodeal nerve induced premature ecdysis behaviour with a shorter latency than is seen using injection of EH. The EH released into the blood is believed to act on the epitracheal glands (Ewer et al., 1997; Kingan et al., 1997) and to account for the other peripheral actions of EH mentioned above.

The role of EH in *Drosophila* has been examined using an expression system to drive expression of cell death genes in the VM neurons ('EH knockouts') (McNabb et al., 1997; Baker et al., 1999). Although elimination of the VM cells (and therefore of EH) produced important defects in ecdysis behaviour in most of the flies, about one third of the flies

completed all ecdyses and emerged as adults. Evidently, EH is not essential for either larval ecdysis or eclosion. Further, knockout flies exhibited a normal circadian rhythm of eclosion., implying that no temporal information is obtained from EH. Jackson et al. (2001) suggest this result indicates the presence of multiple redundant pathways controlling eclosion.

A



B

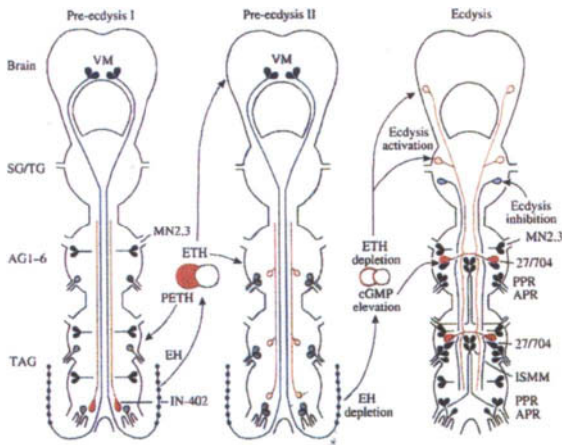


Fig. 5.13. Two models for the hormonal control of ecdysis. In model “A”, the decline in ecdysteroids results in initial release of peptides from Inka cells (blue) and/or eclosion hormone (EH) from ventral medial (VM) neurons (red). The red box is a ‘triggering module’ comprising reciprocal interaction between EH and ecdysis triggering hormone (ETH). The green box is the ‘effector module’ comprising neural inhibition (-) or stimulation (+) of the onset of ecdysis. The width of arrows indicates relative intensity of action while loss of colour from a cell depicts release of its contents. PEI, PEII indicate pre-ecdysis I and II. (From Ewer and Reynolds, 2002). In model “B”, release of EH causes release of ETH and pre-ecdysis triggering hormone (PETH) from Inka cells (red). ETH acts

Fig. 5.13 continued

at several levels in the CNS to coordinate the sequential action of ecdysis behaviour in the chain of ganglia (motor neurons are green). (From Zitnan and Adams, 2000). Both models aim to explain the temporal sequence of events leading to ecdysis after the sequence is activated by appropriate levels of ecdysteroids; thus, no ecdysis clock is involved in either model.

An additional endocrine mechanism involving two peptide hormones from the system of epitracheal glands was discovered by Zitnan et al. (1996) in *Manduca*. Homologous glands were reported in *Bombyx* and *Galleria*. In *Manduca*, there are nine pairs of segmentally repeated epitracheal glands, each composed of three cells. The glands are attached to the trachea adjacent to the spiracle. One of the three cells in each gland (the Inka cell) releases its contents at ecdysis. Extracts of the cells induced premature ecdysis *in vivo* and elicited the neural correlates of the behaviour from isolated abdominal ganglia *in vitro*. Inka cells co-release two peptides, pre-ecdysis triggering hormone (PETH) and ecdysis-triggering hormone (ETH), both of which act on the isolated abdominal ganglia (Zitnan et al., 1999). ETH is also found in *Bombyx* (Adams and Zitnan, 1997). The gene encoding both peptides was found to contain putative response elements for ecdysone, indicating its expression is regulated by ecdysteroids (Zitnan et al., 1999). The *eth* gene of *Drosophila* (Park et al., 1999) encodes peptides that elicit ecdysis in *Manduca*, and the *Manduca* peptides are active in *Drosophila* (Park et al., 1999). The *Drosophila eth* gene also contains ecdysone response elements. As ecdysteroid titres rise during development, Inka cells express the ecdysone receptor and then the *eth* gene. Synthesis of ETH and PETH is induced by high ecdysteroid titres. High ecdysteroid titres were also found necessary for the abdominal nervous system to develop sensitivity to applied ETH. Ecdysteroids also act directly on Inka cells to suppress development of responsiveness to EH (Kingan and Adams, 2000).

It is not yet clear whether release of ETH precedes or follows release of EH. However, the following endocrine events occur at this time. The subsequent decline in ecdysteroid levels induces a commitment of the VM neurons in the CNS to release EH. With further drop in the ecdysteroid titre, EH is released into the circulation; EH induces release of ETH and PETH from the Inka cells. Released ETH exerts a positive feedback on the VM cells, accelerating EH release and promoting final depletion of ETH and PETH from the Inka cells (Ewer et al., 1997; Kingan et al., 1997). PETH and ETH act on the CNS to elicit pre-ecdysis and ecdysis behaviours (Zitnan et al., 1999; Zitnan and Adams, 2000). PETH appears to act on all abdominal ganglia to activate the neural circuitry specific for early pre-ecdysis (pre-ecdysis I in Fig. 5.13). Late pre-ecdysis (pre-ecdysis II) commences 15–20 min later as ETH recruits additional neurons in each abdominal ganglion. The transition from pre-ecdysis to ecdysis requires the brain (Novicki and Weeks, 1996). This transition seems to involve the action of ETH on the brain and/or sub-oesophageal ganglion (Zitnan and Adams, 2000). These anterior ganglia regulate the local release of crustacean cardioactive peptide (CCAP) from segmentally repeated neurons in abdominal ganglia; this centrally released CCAP regulates execution of the ecdysis motor programme (Gammie and Truman, 1997). The relative importance of EH (Ewer et al., 1997; Gammie and Truman, 1999) and ETH (Zitnan and Adams, 2000) in activating the CCAP pathway is currently debated. The above model undergoes extension and revision as new information appears; a critical analysis of current schemes is provided by Ewer and Reynolds (2002). Two current variants are shown in Fig. 5.13. Current debate focuses on whether the sequence of endocrine events is initiated by EH or ETH and the role of the head

(Baker et al., 1999; Gammie and Truman, 1999; Zitnan and Adams, 2000; Ewer and Reynolds, 2002).

From the standpoint of circadian control of ecdysis behaviours, it is clear from the above studies that there exist numerous control points for the timing of ecdysis by ecdysteroids. Low, declining ecdysteroid levels are necessary for the release of EH and for the ability of Inka cells to respond to EH. The CCAP neurons are also potential targets of ecdysteroids (Jackson et al., 2001; Zitnan and Adams, 2000). Collectively, these influences would constrain ecdysis to a very narrow time window, without a requirement for additional clock input. Circadian gating of ecdysis would likely result if the ecdysteroid decline is itself under circadian control. Such mechanisms could account for the observed gating of ecdyses in larvae. Indeed, there is no clear evidence that any of the four peptides involved in the control of ecdysis are involved in the control of its timing in larvae. The additional, direct, circadian clock input at eclosion may derive from input to the VM neurons from clock neurons in the brain. Such additional input might serve to generate direct fine tuning of the eclosion system by increasing the precision of timing with which EH is released at eclosion; this would lead to tighter coordination between the release of EH and ETH and also tighter synchrony among the nine pairs of Inka cells.

Ecdysteroid-centred timing would be expected to deteriorate at the adult ecdysis because the prothoracic glands degenerate around this time. Consequently, the necessary decline in ecdysteroids at the approach of the adult ecdysis may not be regulated with the precision that occurs at larval ecdyses. Yet it is at the adult ecdysis that precision of timing is particularly crucial, as discussed at the beginning of this section. The direct circadian timing of the adult ecdysis may therefore represent a necessary compensation for the degeneration of developmental timing at this crucial moment.

3. Rhythms in cuticle formation

Since the description of 'daily growth layers' in insect cuticle by Neville (1963), a considerable literature has grown up that demonstrates the formation of these layers is regulated by a circadian system. The interest in the subject derives largely from the notion that the structure of the cuticle provides a record of the preceding activities of the epidermal cells that produced it. It is therefore important to understand the nature and origin of the several kinds of layers found in the cuticle prior to discussion of the nature and control of 'daily growth layers'.

The first region of the cuticle to be secreted during a moult cycle is the epicuticle, which itself comprises several layers (Hepburn, 1985). This region is secreted at the time of peak ecdysteroid titres in the blood, during the middle of the moult cycle. The epicuticle is thin, and the great bulk of the cuticle underneath it (procuticle) is deposited afterwards in the presence of progressively decreasing levels of ecdysteroids. Procuticle is only partly formed by the time of ecdysis and its secretion may continue for days or even weeks, being frequently completed during the period of rising ecdysteroid titres that precede the next apolysis. Pre-ecdysial procuticle (loosely, exocuticle) may become morphologically indistinguishable from post-ecdysial procuticle (loosely, endocuticle).

Chitin (n-acetyl glucosamine) is a major component of the procuticle (20 to 50 per cent dry weight). Adjacent chains of chitin are held together by hydrogen bonds into groups known as microfibrils. Chitin microfibrils are about 3 nm in diameter and are embedded in a matrix of protein (Kramer and Koga, 1986). These microfibrils are formed at plaques on the surface of

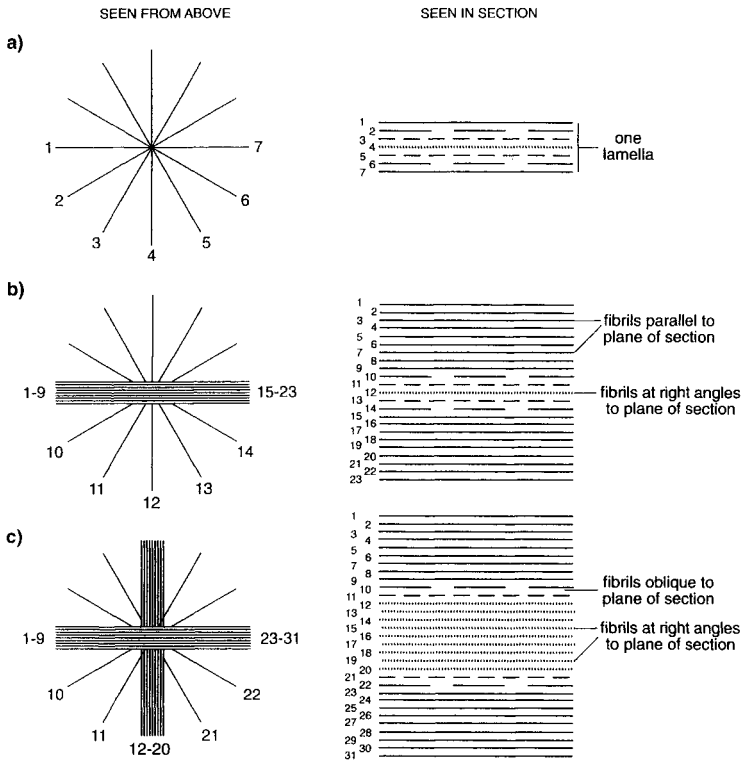


Fig. 5.14. Diagrammatic representation of arrangements of chitin microfibrils in cuticle. Microfibrils lie parallel to each other in the plane of the cuticle but their orientation in relation to each other may vary with depth in the cuticle. The diagrams on the left show the orientation of microfibrils as seen from above and the diagrams on the right show the appearance that would result in a cross section. Numbers indicate successive layers of microfibrils. (a) The microfibrils rotate anticlockwise in a helicoidal arrangement that forms one single lamella per 180° of the helix. (b) Two layers of unidirectional microfibril layers (1-9 and 15-23) form two non-lamellate layers with a single helicoidal lamella (10-14) in the middle. (c) Layers of unidirectional microfibrils at right angles to each other (1-9, 12-20 and 23-31) separated by thin layers with helicoidal arrangements (10-11 and 21-22). (From Chapman, 1998).

the epidermal cells. Proteins are laid down in the spaces between the microfibrils as they form (Neville, 1975). At any particular depth in the formed procuticle, the microfibrils lie parallel to one another in the plane of the cuticle. When viewed from above the cuticle surface, the orientation of the microfibrils in pre-ecdysial procuticle rotates anticlockwise between successive levels within the cuticle. Thus, the orientation of microfibrils changes in a helicoidal pattern when viewed from above (Fig. 5.14a). This arrangement is often referred to as helicoidal 'plywood'. If the cuticle is sectioned vertically, any particular orientation of microfibrils is seen to repeat every 180° of the helix, creating the appearance of lamellae. This helicoidal pattern is known as lamellate cuticle. In the giant water bug, Neville and Luke (1969) found that each lamella (180° twist of the helix) comprised 22 to 25 layers of microfibrils, representing 7° to 8° rotation of the orientation of microfibrils per layer. Thus, the

orientation of microfibrils within the lamellate cuticle changes progressively with depth in the cuticle and therefore with the time of their deposition.

The inner (post-ecdysial) procuticle may have the same structure as the above, resulting in a procuticle that is lamellate throughout. This is seen in Diptera, Coleoptera, larval and pupal Lepidoptera and the Apterygota (Chapman, 1998). In other insect groups, the inner procuticle may have layers of helicoidal (lamellate) cuticle that alternate with layers in which the microfibrils all have a uniform orientation (non-lamellate cuticle). In sections of cuticle, this pattern of the microfibrils gives the appearance of bands of lamellate cuticle alternating with bands devoid of lamellae (Fig. 5.14b and c). One *pair* of such bands constitutes one daily growth layer. Lamellate cuticle is deposited during one part of each day and non-lamellate cuticle is deposited during the remainder of each day (Neville, 1967). Such daily growth layers are found in both larval and adult stages of many hemimetabolous species and in the adult stage of various holometabola (Neville, 1983).

The helicoidal architecture of lamellate cuticle is closely similar to that of cholesteric liquid crystals, implying that it results from extra-cellular self assembly. The helicoids seem to pass through a liquid crystalline state prior to solidifying. For example, a colloidal suspension of chitin fragments displays chiral nematic order and dries to a solid film that mimics the helicoidal organisation of microfibrils in cuticle (Revol and Marchessault, 1993). This helicoidal arrangement is the typical organisation of chitin (GeraudGuille, 1996). Such self-assembling systems are energetically conservative as they require neither enzymatic control nor the formation of high energy chemical bonds (Bouligand, 1978a, b; Neville, 1986). These observations suggest that lamellate cuticle is an energy conservative structure resulting from self-assembly. The self-assembly of chitin into supramolecular helicoids can be assisted or retarded by alteration in conditions such as pH, temperature and electric charge (Murray and Neville, 1997; 1998). Together, these findings indicate that lamellate cuticle may represent the default organisation of microfibrils in cuticle. Some intervention (currently unidentified) would be required to modify the self-assembly process in order to create non-lamellate cuticle. The physiological mechanisms that regulate the formation of post-ecdysial procuticle are essentially unknown (Riddiford, 1985).

It is clear that the alternation between helicoidal and uniform orientation of chitin microfibrils is regulated by a circadian system in some species. For example, rhythmic alternation of the bands in locusts is maintained in DD for at least two weeks (Neville, 1965). In LL, production of helicoids ceased, resulting in a broad, non-lamellate region of cuticle. However, banding persisted in LL when a daily thermoperiod (12 hours at 36° C, 12 hours at 26° C) was provided. The daily banding was restored by a transfer from LL to DD. The period length of the banding rhythm in DD was about 23 hours, so that after 12 days the cuticle bands were 180° out of phase with external time. The period length was temperature-compensated ($Q_{10} = 1.04$). The quantity of material deposited showed conventional temperature sensitivity ($Q_{10} = 2$), resulting in the deposition of *thicker* bands at higher temperatures.

In locust resilin, Neville (1963) demonstrated daily growth layers by the use of ultraviolet light which causes the cross-linking amino acids dityrosine and trityrosine to autofluoresce. Under these conditions diurnal zonation was demonstrated in the prealar arm and the wing hinge ligaments. Confirmation that these layers were in the resilin itself was achieved by labelling the ligaments with tritiated tyrosine at known intervals; this later showed up in autoradiographs as discrete bands at predictable sites (Kristensen, 1966). Each ligament was shown to grow for 2 to 3 weeks after emergence. However, three pairs of layers were found to be deposited before emergence, and these 'pre-imaginal' layers were clearly separated from those subsequently deposited (the 'post-imaginal' layers) by a pause in cuticle formation

called the 'emergence line'. In contrast to the deposition of solid endocuticle the rhythmic formation of resilin persisted slightly in LL and seemed to be mainly influenced by the temperature cycle.

Neville (1967) reported that the rhythm of cuticle deposition in locusts continued when their head capsules were painted black, or when their eyes and ocelli were removed by cautery. The site of photoreception, therefore, is not in the head. Furthermore, cylinders of the hind tibiae cut from living specimens of *Schistocerca gregaria* continued to form growth layers when implanted into the haemocoel, demonstrating that neither mechanical nor nervous connections were required. Neville (1967) showed that the daily growth layers of *Schistocerca gregaria* and *Locusta migratoria* could be uncoupled from the circadian clock by exposure to LL and constant temperature, as evidenced by loss of banding in the cuticle that was deposited during aperiodic conditions. When one hind tibia was painted black prior to transfer to these aperiodic conditions, rhythmicity in cuticle deposition was lost in both the painted and unpainted legs. Neville surmised this result was caused by light leaking through the paint and repeated this experiment with the difference that five locusts were transferred from LD to very dim LL and a 7° C lower temperature (5 ft-c; 27° C). In this case, the painted tibiae deposited banded cuticle for all 8 days, whereas the unpainted ones deposited only non-lamellate cuticle. This differential response of painted and unpainted legs was interpreted as evidence that light is measured directly by the epidermal cells. The remarkable result in this experiment is that the *unpainted* areas of the locust were said to deposit unbanded cuticle, meaning that the massive drop in light intensity from 150 to 5 ft-c was not registered as 'lights-off' and 5 ft-c was *not* interpreted as relative darkness. The stated differential response between painted and unpainted legs of the same animal was said to eliminate the involvement of nervous and endocrine control and suggested local photoreception such as an epidermal light sense. However, some epidermal cells continue to produce banded cuticle in LL (Neville 1965), suggesting discrete photoreceptors might exist in either the epidermis or adjacent leg tissue. An action spectrum for the response was conducted in intact *Schistocerca gregaria* and showed a peak in the region of 470 nm, consistent with a carotenoid chromophore. Carotenoids contribute to the colour of the locust integument. In *Oncopeltus fasciatus*, Dingle et al. (1969) found the rhythm to be entrained by the light cycle experienced by the pharate adult. In this species the rhythm is also temperature-compensated (Q_{10} about 1.0). Interestingly, the total thickness of the cuticle is also temperature-compensated; at higher temperatures, thicker layers are formed each day, but cuticle secretion ceases after fewer days. For example, the final cuticle thickness of 30µm was achieved in 10 to 11 days at 19° C; at 27° C, this thickness was reached in 5 to 9 days.

Zelazny and Neville (1972) showed that the deposition of the endocuticle in *Oryctes rhinoceros* and some other beetles, although rhythmic, was controlled by a non-circadian system. Firstly, there was no linear correlation between age and the number of layers deposited. In beetles maintained in DD since eclosion, the number of layers increased steadily and then remained constant: in *Oryctes rhinoceros*, for example, about twenty-two layers were evident in the pronotum after about 40 days. Secondly, the frequency of layer formation was higher at 34° C than at 24° C, and more endocuticle was deposited. However, the Q_{10} for the number of layers deposited (in 12 days) was 1.8, whereas that for the increase in thickness was only 1.46; consequently individual layers became thinner at the higher temperature. Thus, the period of the oscillation in *Oryctes* differs from 24 hours; the oscillation will not entrain to environmental cycle; and it is not temperature-compensated. Moreover, the period length seemed to vary between regions of the integument. In these beetles, the deposition of layered cuticle is not merely non-circadian, it seems to be not timed.

The formation of banded cuticle has been studied extensively in cockroaches, where both similarities and differences are seen from the picture developed using locusts. Banded cuticle is deposited for about 14 days after the adult ecdysis in *Blaberus craniifer* and *Leucophaea maderae*, with lamellate cuticle being formed during the day, as distinct from the night in locusts. Runte and Weber (1982) note that lamellate cuticle is formed during the portion of the day when the insect is behaviourally active in both cases. The bands are formed with circadian periodicity that is temperature compensated (Q_{10} about 1.1) (Weidenmann et al., 1986). The fundamental features of circadian control are therefore evident. However, the rhythm remained daily in LL, DD and in non-circadian light cycles such as LD 6:6, 10:10, 13:13 and was synchronous in legs covered with aluminium foil and exposed legs of the same animals (Weidenmann et al., 1986). Therefore, the rhythm of cuticle deposition in both species seems to be unaffected by light. Neville (1965) had previously reported that the cuticle rhythm of the American cockroach, *Periplaneta americana* persisted in LL.

It is not known whether the rhythm can be entrained by temperature cycles in any cockroach. Even if it is, neither light nor temperature cues appear necessary for rhythmicity; when *Blaberus* or *Leucophaea* were transferred to LL up to 23 days prior to the adult ecdysis, banded cuticle secretion commenced at ecdysis and continued in daily fashion thereafter (Weidenmann et al., 1986). It was inferred that a signal associated with ecdysis initiated the banding rhythm, which was then maintained under apparently aperiodic conditions. Since the onset of rhythmicity was synchronous throughout the animal, a hormone released at ecdysis appears to be responsible for initiation of the rhythm (Lukat et al., 1989). However, in locust resilin (Kristensen, 1966) and the wasp *Paravespula vulgaris* (Lermette, 1977) several daily bands are present immediately after ecdysis, indicating that any such signal may occur prior to ecdysis. Hormonal signals appear to be unnecessary for the maintenance of banding following initiation; leg pieces of young adult cockroaches which were implanted into the haemocoel of old hosts in which cuticle secretion had ceased continued to deposit banded cuticle (Lukat et al., 1989). The central nervous system likewise seemed unnecessary for maintenance of banding; decapitation, bilateral removal of optic lobes, removal of the thoracic or abdominal ganglia all had little influence on the banding rhythm (Lukat et al., 1989). The animals without optic lobes showed arrhythmic locomotion. Further, pulses of light in DD that phase-shifted the locomotor rhythm were without affect on the banding pattern (Weidenmann et al., 1986). Clearly, the cuticular banding rhythm is independent of the clock controlling locomotion. It seems that the banding rhythm in cockroaches is driven by light-insensitive circadian oscillators in peripheral tissues (possibly epidermis) that are turned on by a hormonal signal. How they are sustained and eventually turned off is unknown.

There is evidence of a molecular oscillator (see Chapter 4) in various peripheral tissues of *Drosophila* (Plautz et al., 1997). Rhythmic *per*-driven bioluminescence was seen in various tissues including the thorax, legs and wings. These rhythms were light-entrainable *in vitro* and free-ran in DD for 2 to 3 cycles. The thoracic apodemes of *Drosophila* exhibit daily growth bands (Johnson and Ellison, 1982). One function of *per*-based peripheral oscillators might be to drive rhythms of cuticle banding. However, the general body cuticle of *Drosophila* does not show daily growth bands.

In several species mentioned above, the rhythm of cuticular banding seems to be initiated by hormones at or before ecdysis. Such banding may continue after ecdysis without further signals and may even be unresponsive to them. These features are consistent with the possibility that cuticular banding may represent a legacy of rhythmicity in the epidermal cells that was established and driven by rhythms in ecdysteroids prior to ecdysis. In other words,

epidermal cells may retain rhythmicity in the adult for some time after their normal driving input (ecdysteroids) has ceased.

4. Egg hatching

‘Egg hatching’ refers to the behaviour by which the first instar larva emerges from the egg case. It consists of chewing movements of the mandibles and co-ordinated twisting movements of the body. Hatching is therefore the first complex behaviour manifest in the life of an insect. Hatching occurs once the first instar larva is developed, which implies that the onset of hatching behaviour is coordinated with the completion of embryonic development.

In numerous species, hatching is a gated event that is under circadian control (see Chapter 3). In many species the hatching rhythm can be entrained by light and/or temperature cycles to which the developing embryo is exposed, showing that both receptors and clock are differentiated and functional prior to hatching. The time of hatching is not a function of the time of oviposition. Rarely, there is evidence of a maternal factor in the egg that influences the time of hatching (Bateman, 1955; Ito and Sumi, 2000). Interest in hatching rhythms has revolved around the related questions of when during embryogenesis the ability to perform circadian timekeeping develops, and how the onset of hatching behaviour is synchronised with embryonic development.

In order to provide a context for this literature, some key events in the differentiation of nervous and endocrine systems will be summarised. The timing of events in embryology is usually referred to the percentage of the total embryonic period from oviposition to hatching. The use of percentages facilitates comparison between species with different total embryonic times. In *Drosophila melanogaster*, neuroblasts develop from the ectoderm at about 20 per cent embryonic development (Doe and Goodman, 1985). These cells differentiate into neurons, glial cells and sheath cells, which start to appear at about 30 per cent (Thompson and Siegler, 1993); the neurons then develop growth cones which elongate into axons. Synapses develop at about 70 per cent in *Locusta* (Leitch et al., 1992), after which action potentials become detectable. Prothoracic glands begin to differentiate at 40 per cent in *Locusta*, but it is not known if the glands are able to secrete ecdysteroids prior to hatching (Lageux et al., 1979). Most insects undergo several embryonic moults within the egg prior to hatching (Hoffman and Lageux, 1985), associated with the secretion of several (usually three or four) embryonic cuticles. The first cuticle to be formed during embryonic development is the serosal cuticle, which is secreted by the blastoderm at about 20 per cent. The first embryonic cuticle is secreted in many insects at about 35 per cent development, and is broken down and replaced by a second embryonic cuticle at about 45 per cent. The cuticle of the first instar larva starts to be formed at about 75 per cent. These embryonic moults are believed to be regulated by ecdysteroids just as in post-embryonic development (Hoffman and Lageux, 1985). Ecdysone and 20E are obtained by the embryo from conjugated ecdysteroids bound to yolk proteins (Sall et al., 1983). Thus, the embryo is provided with a *maternal* supply of inactive ecdysteroids, from which it obtains the free hormones at appropriate intervals. Diptera do not form embryonic cuticles prior to that of the first larval stage at about 75 per cent.

Truman et al. (1981) found that *Hyalophora cecropia* embryos contained material that was active in eclosion hormone (EH) bioassays. The amount of this material dropped at the embryonic ecdyses, but not at hatching. Fugo et al. (1985), however, obtained converse results in *Bombyx mori*: EH-like activity did not fluctuate during embryonic moults but dropped abruptly following hatching.

Several studies have examined the time during development at which the circadian clock that controls hatching becomes functional. Minis and Pittendrigh (1968) transferred batches of developing eggs of the boll worm moth *Pectinophora gossypiella* from LL to DD at numerous points during embryonic development. They obtained rhythmic hatching from eggs transferred to DD at 50 to 60 per cent development or later (see also Chapter 3). Transfers earlier to this point resulted in arrhythmic hatching. Very similar results were obtained for *Antheraea pernyi* (Sauman et al., 1996) and *Gryllus bimaculatus* (Tomioka et al., 1991; Itoh and Sumi, 2000). These findings show a photosensitive clock that controls hatching becomes functional at about 50 to 60 per cent development. The arrhythmic hatching of eggs transferred before this point in development could be due to lack of functionality in either the light entrainment pathway or the clock itself. Minis and Pittendrigh (1968) addressed these two alternatives in *P. gossypiella* by using temperature rather than light as the *Zeitgeber*. They found that entrainment to temperature cycles was first possible at about the same time that light entrainment became possible, indicating that both clock and entrainment pathways became functional at about the same time. It is noteworthy that this time is close to (slightly before?) the first appearance of synapses and action potentials in the nervous system (see above). Bruce and Minis (1969) exposed eggs to various wavelengths of monochromatic light after mid-embryogenesis and found that the hatching rhythm of *P. gossypiella* was maximally sensitive to wavelengths of 390 to 480 nm, consistent with a carotenoid-based photoreceptor. Sakamoto and Shimizu (1994) reared *Bombyx mori* on a carotenoid-deprived artificial diet and examined rhythmicity of hatching by the carotenoid-depleted eggs. The photosensitivity of the hatching rhythm was essentially normal. By contrast, ocellar photosensitivity was reduced by four orders of magnitude and newly hatched first instar larvae showed suppressed phototaxis. This finding might suggest that the light input pathway for the hatching rhythm is different from that for the locomotion rhythm.

Sauman et al. (1996) found that immunoreactivity to the period protein (PER) first appeared in four pairs of cells in the brain of *Antheraea pernyi* at the time that the hatching rhythm became entrainable (i.e. 50 to 60 per cent development). However, staining was exclusively cytoplasmic and did not show daily cycling, as usually occurs in circadian 'clock cells' (see Chapter 4). At 60 to 70 per cent development, PER immunoreactivity appeared in cells of the midgut; in this location, nuclear cycling of PER reactivity was seen that was found to free-run in DD. PER protein was found to be necessary for rhythmicity in hatching; embryos injected with an anti-sense oligonucleotide of *per* at one day before hatching subsequently hatched at random. Controls injected with a reverse orientation anti-sense oligonucleotide of *per* remained rhythmic. Thus, PER is required for rhythmic hatching. The relative roles of brain and midgut in regulation of hatching were then examined (Sauman and Reppert, 1998). Brains were removed one day before hatching and implanted into recipient embryos that were entrained to a light cycle that was phase delayed by 8 hours relative to the brains (Fig. 5.15). Hatching was then monitored in DD. It was found that the implanted brain advanced the phase of hatching by 8 hours; i.e. the implanted brain dictated the phase of hatching. Clearly, the brain releases a humoral factor that regulates timing of hatching within a day (i.e. the hatching gate). Transplants of midgut tissue did not affect the hatching gate. It was inferred that the clock for hatching resides in the brain and is associated with the PER-positive cells found therein. These PER-positive cells were considered to correspond to the PER-positive cells found in the brain of adult *Antheraea pernyi* (Sauman and Reppert, 1996), where they appear to regulate release of PTTH (see Section C1a). It is interesting to note that the PTTH-producing cells in the brain of *Manduca sexta* contain immunoreactive 'big' PTTH from 24 to 30 per cent of development onwards (Westbrook and Bollenbacher, 1990) and that embryos of

M. sexta contain biologically active PTTH (Dorn et al., 1987). It is therefore conceivable that the 'PTTH clock' of post-embryonic life also plays a role in the control of hatching.

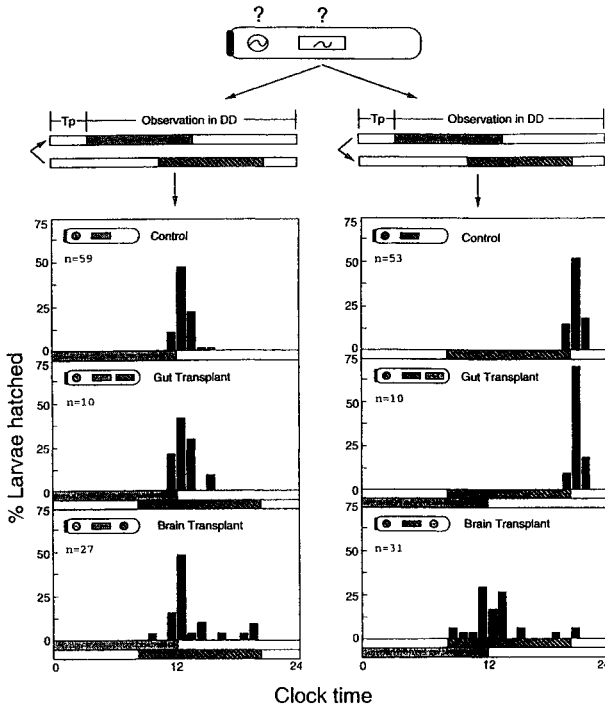


Fig. 5.15. Experiments that show that a diffusible factor from the brain of developing larvae of the silkworm, *Antheraea pernyi*, controls the circadian gate of egg-hatching behaviour. Brain and midguts were transplanted into host embryos that were entrained to a light cycle that was 8 hours advanced or delayed relative to that of the donor embryos. Following surgery, the embryos were transferred to DD and allowed to hatch. The panels on the left show that when donor gut (middle panel) or donor brain (lower panel) originate from embryos that are 8 hours phase delayed relative to the host, the time of hatching of the host is not altered from controls (top). This indicates that the intrinsic brain activates hatching at the normal time and the release of any factor by the implanted tissues 8 hours later is redundant. The converse design is shown in the right hand panels, where implanted gut or brain are phase advanced by 8 hours relative to the host. Here, the gut implant has no effect (compare top two panels), whereas the implanted brain (bottom panel) causes hatching to occur 8 hours earlier than controls. Thus, the implanted brain releases a factor that times hatching. It can be detected when released earlier than that of intrinsic brain but not if released later. See text for details. (From Sauman and Reppert, 1998).

In *Antheraea pernyi*, the brain is also necessary for the cycling of PER in the midgut (Sauman and Reppert, 1998), but this control appears to require nervous connections between the brain and midgut. The function of the embryonic gut oscillator is unknown. It is possible that it is involved with yolk uptake and/or uptake of maternal ecdysteroids from the yolk during embryonic moults. These considerations suggest a possible pathway by which the brain might regulate ecdysteroids during embryonic moults.

Melatonin, which is known to be an important element in circadian and photoperiodic systems in vertebrates, is under circadian control in embryos of the cricket *Gryllus bimaculatus*

(Itoh and Sumi, 1998a, b)(see also Chapter 14). Freshly laid eggs contain abundant melatonin; this is presumably of maternal origin since ovaries of crickets also contain this indoleamine (Itoh et al., 1994; 1995). The level of melatonin in eggs begins to show a daily rhythm on day 3 (about 20 per cent development) (Itoh and Sumi, 1998a). The enzyme responsible for rhythmicity in melatonin synthesis in vertebrates is N-acetyl transferase (NAT). NAT activity appears in eggs of *G. bimaculatus* on day 3 and was shown to free-run in DD by day 6 to 7 (about 40 per cent development) (Itoh and Sumi, 1998a) (Fig. 5.16). Thus, circadian regulation of NAT activity, and by implication melatonin synthesis, is established by mid-embryogenesis. The function of melatonin is not known at any stage of the insect life cycle. The importance of this hormone in timekeeping systems of other animals suggests that further work with melatonin should be fruitful.

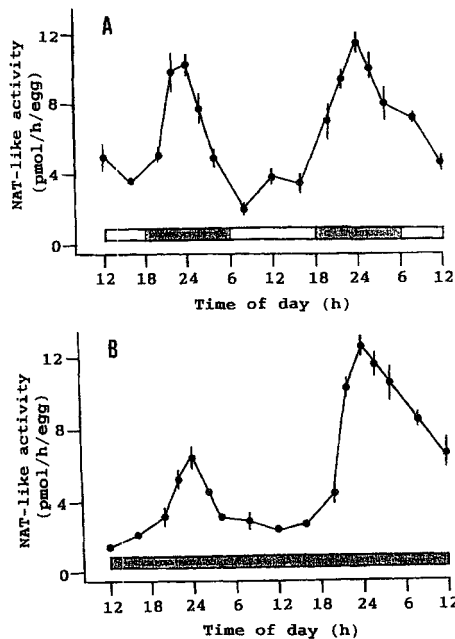


Fig. 5.16. Entrained rhythm (A) of N-acetyltransferase activity in the eggs of the cricket *Gryllus bimaculatus* during days 6 and 7 of embryogenesis. The rhythm free-runs in DD (B). See text for details. (From Itoh and Sumi, 1998a).

In conclusion, various lines of evidence have shown that circadian organisation in insects first becomes apparent about half way through embryonic development; the first overt event known to be under circadian control is the hatching behaviour of the first instar larva from the egg. The known association (above) of hatching with PTTH and PER cells in the brain suggests the possibility that these experiments define the point in embryonic development when the central timing system (Section C1c) becomes functional.

D. CIRCADIAN ORGANISATION IN ADULTS

The physiology of adult insects is organised around cycles of reproduction. Reproductive cycles are not endogenously timed; they are heavily dependent on external variables such as the availability of food. However, they can be turned on or off by photoperiodic cues, as in reproductive diapause (see Chapter 9). But within each reproductive cycle, the sequence of physiological events is organised into a highly structured sequence. The sequence begins with the production and maturation of eggs and sperm; these processes must be synchronised between males and females for reproduction to be successful. They must also precede attempts by either sex to locate a mate by stridulation or release of pheromones. Courtship, mating, egg fertilisation, location of an oviposition site and then oviposition must all follow in precise sequence. Thus, the constituent events within a reproductive cycle are organised into a specific temporal sequence within an individual insect. Many of these events have been shown to be subject to circadian control; it is quite possible that circadian organisation pervades the entire cycle. In addition, successful reproduction requires synchrony among the male and female members of an insect population; when individuals within the population consult a common environmental *Zeitgeber*, they become synchronised with each other, forming a population that passes in synchrony through the stages of the reproductive cycle.

The overall structure of events within a reproductive cycle is governed by reproductive (and other) hormones and thus by the neuroendocrine system. The great variety of reproductive strategies employed by insects has resulted in an equally great variety of neuroendocrine control mechanisms; this variety precludes the presentation of a generalised scheme. However, it should be noted that the same basic group of hormones and nervous pathways is found in all insects; it is their relative importance and detailed function that seems to vary. The absence of a general scheme for the physiological regulation of reproduction requires that the information below is presented separately for the various phenomena that are known to be subject to circadian control. In some cases, there is information regarding the neuroendocrine pathways and the mechanisms by which overt rhythms are controlled. These instances encourage the expectation that commonalities in regulatory pathways may soon be found, which will lead in turn to discovery of how the various adult rhythms are coordinated.

1. Circadian regulation of adult hormones

The organisation of adult rhythms around and within cycles of reproduction raises the prospect that the reproductive hormones themselves may be central to the circadian organisation of adult life. All the developmental hormones discussed above (Section C1) as central to the circadian organisation of larvae persist into the adult stage, where they may be equally important in circadian organisation. Some evidence that points to functions of these hormones, particularly *juvenile hormone*, in the circadian organisation of adult insects is presented below.

Circadian regulation of PTTH appears to persist in adult insects. PTTH was initially purified from adult heads of *Bombyx* (Ishizaki and Suzuki, 1994); it is found at high levels in adult haemolymph of *Bombyx* (Mizoguchi et al., 2001) and brains of *Rhodnius* both contain and release PTTH (Vafopoulou et al., 1996). Further, the PTTH cells of *Rhodnius* (Steel and Nseiri, 2002) and their associated 'clock neurons' (Terry and Steel, 2002) persist into the adult. Similarly, in adult *Antheraea*, both the PTTH cells and the adjacent circadian 'clock neurons'

that appear to regulate PTTH release also persist into the adult stage (Sauman and Reppert, 1996). Thus, both PTTH and the machinery for its circadian regulation are present in adults. It is conceivable that PTTH is as central to the circadian organisation of adult insects as it is to larvae. The function of PTTH in adults, however, is currently unknown.

The PGs degenerate within a few days of adult emergence. The main target of PTTH in larvae is therefore absent in adults. However, ecdysteroids are not absent in adults, being produced by the testes or ovaries (Hagedorn, 1989), where they are said to participate in various aspects of egg and sperm production and are regarded as the sex-steroids of insects (DeLoof et al., 2001). The ecdysteroid levels in the haemolymph are much lower in adults than in larvae and whether these levels are rhythmic remains uncertain.

The juvenile hormones (JHs) are synthesised and released from the *corpora allata* (CA) in both larvae and adults. JHs are regarded as central regulators of reproduction (Koeppel et al., 1985) and have been studied extensively by endocrinologists. It is only recently that evidence is emerging that JHs may play an equally important role in circadian organisation. Evidence is accumulating that JH synthesis by CA is rhythmic, is under the control of 'clock cells' in the brain and is intimately involved in the expression of numerous circadian rhythms. Some of this evidence is presented below.

JHs are small, hydrophobic hormones that are released from the CA as they are synthesised. Release from the CA is therefore governed primarily by the rate of synthesis. Synthesis of JHs by the CA is regulated by nerves from the brain, which include neurosecretory neurons containing peptide *allatotropins* and *allatostatins* (Stay et al., 1994). In *Manduca sexta*, there are two groups of neurons in the brain that are immunoreactive for allatostatins and possess numerous axon terminals within the CA (Homberg et al., 1991; Zitnan et al., 1995). Similarly, in *Drosophila melanogaster* Siegmund and Korge (2001) identified two groups of secretory neurons in the protocerebrum that terminated at synaptic boutons in the CA (see Fig. 5.6 above). Both of these groups of cells in *Drosophila* have dendritic fields that overlap with, and presumably receive synaptic input from, the lateral 'clock neurons' in which the 'clock genes' *per* and *tim* cycle (see Chapter 4). Thus, the innervation of the CA receives input from the lateral 'clock neurons'. In the moth *Antheraea pernyi* (Sauman and Reppert, 1996), two separate groups of neurons expressing *per* and *tim* are present in the protocerebrum. In both groups, PER protein was identified in the cytoplasm, enabling the axonal projections of these cells to be traced. The axons of both groups of cells were found to terminate in the CA. Thus, a neural pathway is present in both *Drosophila* and *Antheraea* that connects the CA with identified 'clock neurons' in the brain, indicating that the CA receive circadian input. This input may be either direct or by regulation of allatotropin/allatostatin cells in the brain.

A further mechanism that may regulate rhythmicity in circulating JH levels is clock-controlled availability of JH binding proteins (JHBP) in the haemolymph. JH travels in the haemolymph attached to JHBP partly to protect it from haemolymph esterases and partly because JH has low water solubility. Thus, the availability of JHBP is necessary for JH transport to target tissues. In *Drosophila melanogaster*, the gene *take-out* (*to*) is a clock controlled gene that is transcribed with a circadian rhythm (So et al., 2000). *to* transcript levels free-run in DD (see Chapter 4). The significance of this finding to JH is that the protein encoded by *to* (TO) is a member of a superfamily of insect proteins that includes both the haemolymph JHBPs of *Manduca sexta* and *Heliothis virescens* and the nuclear JHBP of epidermal cells of *Manduca sexta* (Touhara and Prestwich, 1992; Touhara et al., 1993; Wojtasek and Prestwich, 1995). These findings suggest that TO may be a JHBP. It is therefore

possible that JH transport and/or nuclear action may be regulated by the circadian availability of JHBP.

In the honey bee, *Apis mellifera*, the transition from nursing behaviour to foraging behaviour is accompanied by the onset of a daily rhythm in JH levels in the haemolymph (Elekonich et al., 2001) (Fig. 5.17). Nurse bees show no apparent rhythm in JH titre. Forager bees possess a marked circadian activity rhythm, which is also absent in nurses; foragers that revert to nursing behaviour undergo both a drop in JH titre and loss of the activity rhythm (Bloch and Robinson, 2001). Thus, behavioural rhythmicity seems to be closely linked to rhythmicity in JH. Moreover, *per* transcription in the brain increases in both amount and amplitude of cycling with the transition to foraging (Toma et al., 2000), consistent with the pathways discussed above for control of JH by 'clock cells' in the brain. Together, these findings indicate that rhythmicity in JH titres do occur and may be driven by the brain. Further, rhythmicity in JH titre seems to be linked to expression of the circadian activity rhythm.

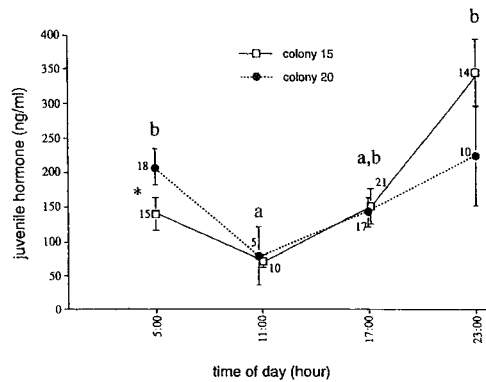


Fig. 5.17. Daily changes in the haemolymph JH titre in two different colonies of foraging honey bees, *Apis mellifera* L. The level of JH in the haemolymph is high during the night, while it is significantly lower during the day. (From Elekonich et al., 2001).

A similar relationship between JH and behavioural rhythmicity is seen in the cockroach *Blattella germanica*. In males, the circadian locomotor rhythm persists throughout adult life (Leppa et al., 1989). By contrast, behavioural rhythmicity in females is modulated in relation to the gonadotropic cycle. Newly emerged females exhibit a locomotor rhythm, but when they become sexually receptive, rhythmicity is lost and locomotion occurs throughout the day (Lee and Wu, 1994). Following expulsion of the ootheca, locomotion ceases for a few days and then again becomes rhythmic as the second gonadotropic cycle begins. Thus, rhythmic locomotion is confined to times of expected high JH production. Sexually receptive females that showed continuous locomotion were ovariectomised (Lin and Lee, 1996); these animals develop a clear circadian locomotor rhythm that free-runs in DD (Fig. 5.18). The authors inferred that the ovary produces a factor that masks expression of the locomotion rhythm. Allatectomy of these ovariectomised females disrupted the circadian locomotor rhythm and reduced the level of activity (Lin and Lee, 1998). This finding again implies that JH is involved in expression of the locomotor rhythm. Topical application of a JH analogue restored the level of activity but not rhythmicity. Topically applied JH enters the haemolymph continuously over many days and

therefore provides a continuous, not rhythmic, supply of JH. It is necessary to determine whether JH synthesis is rhythmic in order to clarify the role of JH in behavioural rhythms.

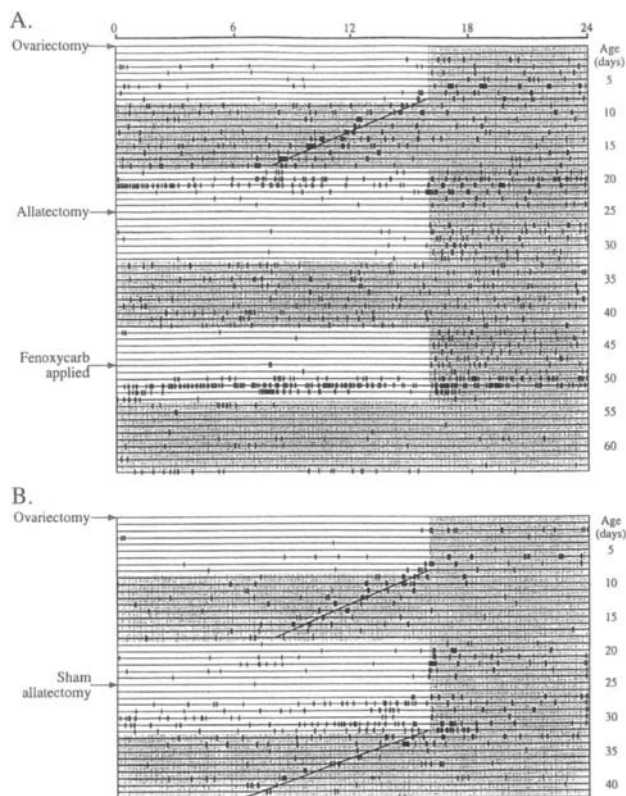


Fig. 5.18. Free-running activity rhythm of ovariectomised female German cockroaches, *Blattella germanica* L. Allatectomy (A), but not sham operation (control; B) disrupts this circadian activity rhythm and reduces the overall level of activity. Topical application of the JH analogue, fenoxycarb, to allatectomised animals restores the level of activity but not the circadian rhythm (A). (From Lin and Lee, 1998).

JH also plays a role in the regulation of *pheromone* production in moths (see also Section D3c). In *Agrotis ipsilon*, pheromone production (Picimbon et al., 1997), the amount of pheromone in the pheromone gland and pheromone release during calling behaviour (Gemeno and Haynes, 2000) all exhibit daily rhythms. Allatectomy of newly emerged females abolishes the calling behaviour; the pheromone glands of these allatectomised females neither contained nor released pheromone (Picimbon et al., 1995). This effect is not solely due to interference with the gonadotropic cycle, since significant levels of pheromone biosynthesis-activating neuropeptide (PBAN) were found in the brains and SOG of the allatectomised animals. Production of pheromone could be restored by injection of PBAN, suggesting that endogenous PBAN was not released in allatectomised animals. This view was confirmed by the finding that injection of JH III restored pheromone production in allatectomised females, but had no effect on decapitated animals. Thus, JH is necessary to the release of PBAN and may regulate

pheromone production by promoting PBAN release at precisely gated times (Picimbon et al., 1995). Similar findings were obtained using *Helicoverpa armigera* by Fan et al. (1999). Pheromone production and levels of PBAN are also rhythmic in this moth (Rafaeli et al., 1991; see also Section D3c). Treatment of newly emerged females with JH induced pheromone production by the pheromone gland. However, neither JH nor PBAN induced pheromone production in newly emerged, decapitated females; it was necessary to inject both hormones together to obtain stimulation of pheromone production. *In vitro* experiments confirmed that both JH and PBAN were needed together to stimulate pheromone production by glands of pharate adults. These findings could signify a role of JH in priming the PBAN system during gonadotropic development, but might equally signify involvement of JH in the regulation of rhythmicity of pheromone production.

Males of *Agrotis ipsilon* respond to female pheromone with upwind flight behaviour. Allatectomy of young males abolished this response (Gadenne et al., 1993; Duportet et al., 1996). However, the antennal sensory system of allatectomised males was found to be sensitive to pheromone, which implied that JH mediated these changes in responsiveness to pheromone through a central, rather than peripheral mechanism (Gadenne et al., 1993). Olfactory information is integrated in the antennal lobe of the brain. Intracellular recordings from antennal lobe interneurons revealed a decreased central response to pheromone in allatectomised males (Anton and Gadenne, 1999; Gadenne and Anton, 2000). JH injection restored the responsiveness of these neurons. It is now clear that male responsiveness to pheromone oscillates with a circadian rhythm in this species (Fig. 5.19) (Gemeno and Haynes, 2000) (see Section D4). The possibility is thereby raised that the JH-mediated changes in central responses also occur daily, possibly signalling that JH also undergoes rhythmic daily changes.

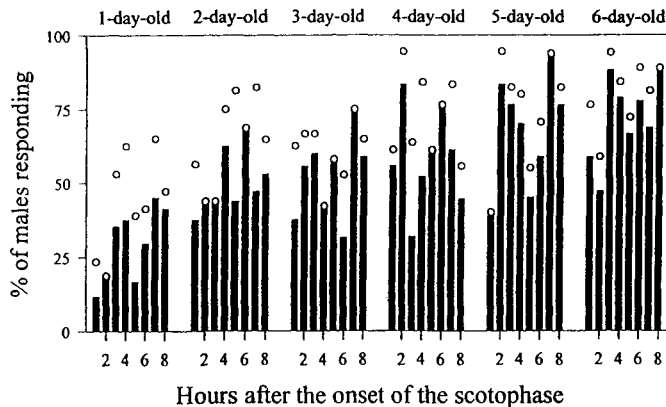


Fig. 5.19. Daily rhythms of responsiveness of males of the black cutworm moth *Agrotis ipsilon* (Hufnagel) to females releasing sex pheromone. Dots indicate the percentage of males orienting to females and bars indicate the percentage of males making contact with females. (From Gemeno and Haynes, 2000).

A further example of JH-mediated rhythmicity in sensory interneurons is found in the cricket *Acheta domesticus*. In males of *Acheta*, the rhythm of stridulation is under circadian control (Rence et al., 1988). The response of females to male stridulation, and therefore mating, is regulated by JH (Atkins and Stout, 1994; Koudele et al., 1987; Stout et al., 1998). Allatectomy of females reduced both the directionality of the phonotactic behaviour and sexual

responsiveness, and both were restored by topical application of JH (Atkins and Stout, 1994; Koudele et al., 1987). It was concluded that the changes in phonotactic thresholds were regulated by changes in JH levels. JH appeared to exert this effect by regulating the threshold for firing action potentials in auditory interneurons in the prothoracic ganglion. Topical application of JH to the prothoracic ganglion, or injection of JH directly into the ganglion, reduced both the firing threshold of the interneurons and the loudness of a mock calling song required to elicit female phonotactic behaviour. Therefore, JH appears to act directly on the neurons in the prothoracic ganglion. Similar JH applications to other ganglia were ineffective (Stout et al., 1991). Moreover, the action of applied JH was seen within 2 hours. Indeed, Stout et al. (1998) report a daily rhythm in the threshold for phonotactic behaviour, but only in 3 day old crickets. These data suggest that a rhythm in JH levels may underlie the observed changes in phonotactic threshold.

Taken together, the studies above suggest that rhythmicity in JH levels may be widespread in adult insects. Such rhythmicity could be a major factor in coordination of the various rhythms associated with reproductive processes. Further, hormones which are known to be under circadian control in the larval stages (PTTH, ecdysteroids) are present in adult insects, as are the neuronal pathways that mediate this circadian control. Therefore, it seems likely that circadian regulation of these core hormones (JH, PTTH, ecdysteroids) will be found to have a central role in the regulation of the rhythmicity of the diverse processes associated with reproduction. It is possible that the putative central timing system for development continues to function in the adult through circadian control of the same hormones. At the present time, evidence of a central coordinating mechanism underlying reproductive processes in general, and the numerous constituent phenomena that are clearly under circadian control, is spectacularly lacking.

2. Gamete formation and transport

The above section illustrates that both development and reproduction are regulated by a common set of hormones. The existence of circadian rhythmicity in these hormones during development raises the prospect that similar rhythms participate in the regulation of reproduction. It should also be noted that the maturation of the reproductive system, and often the formation of mature eggs and sperm, occurs during late larval life under the control of these developmental hormones (review by Hoffman, 1995). Both ecdysteroids and JH act directly on the reproductive system. The role of ecdysteroids has been especially noted in a variety of Lepidopteran species (Gillott, 1995). In species when the adult is short-lived, the complete complement of mature gametes may be present at the time of the adult ecdysis. The central importance of both ecdysteroids (Hagedorn, 1985, 1989; DeLoof et al., 2001) and JH (Koeppel et al., 1985; Wyatt and Davey, 1996) in the regulation of gametogenesis in both males and females is very heavily documented. In adult insects, ecdysteroids are secreted by both ovaries and testes and are regarded as the insect sex steroid hormones (DeLoof et al., 2001).

Gametogenesis can be arrested in either sex by withdrawal of these hormones, as occurs in reproductive diapause (Denlinger, 1985). Reproductive diapause is frequently under photoperiodic control (see Chapter 9 et seq.). Therefore, it is apparent that central clock control of these hormones is employed to regulate gametogenesis. In other words, periodicity in gametogenesis is achieved by clock control of reproductive hormones. These considerations, coupled with evidence of rhythmicity in these same hormones in adults (Section D1) leads to the

expectation that gametogenesis itself might be rhythmic. Indeed, rhythmicity in gametogenesis might be a central phenomenon that could result in rhythmicity in a wide variety of other events associated with reproduction. There is a paucity of information concerning rhythmicity in gametogenesis that reflects a dearth of studies rather than absence of the phenomenon.

(a) Oogenesis

The possibility of rhythmicity in oogenesis has been raised in the literature only concerning *Drosophila*, but the evidence is unclear. Allemand (1976a,b) reported that in *Drosophila* the frequency of egg chambers in various stages of development appeared to vary with the time of day that ovaries were dissected. The changes were described as a rhythm in vitellogenesis, specifically in the time of onset of stages 8-10 of vitellogenesis. The rhythm was said to free-run in DD for at least five days. The endocrine factors that regulate vitellogenesis in *Drosophila* are heavily studied; both ecdysteroids and JH are involved in the regulation of vitellogenesis in *Drosophila*. The complexity of this regulation is shown in Fig. 5.20.

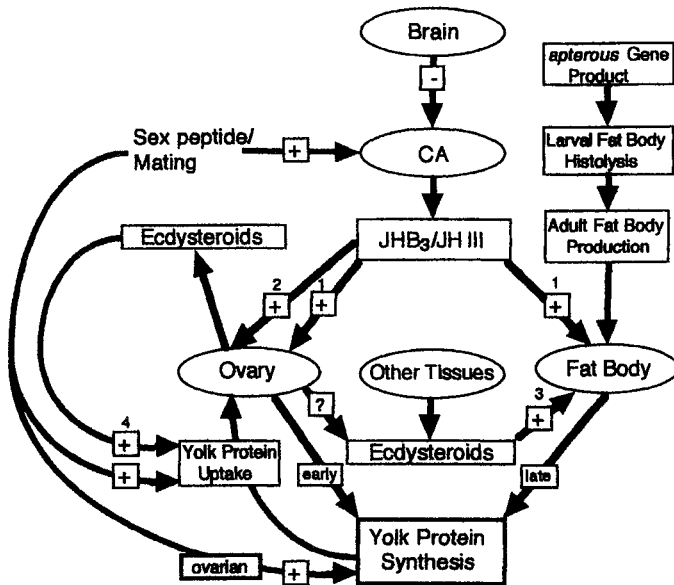


Fig. 5.20. Scheme showing the complexity of regulation of vitellogenesis in females of the fruit fly, *Drosophila melanogaster*. Both JH from the corpora allata and ecdysteroids from the ovaries are involved in this regulation and act on both the fat body and ovaries. (From Richard et al., 1998).

If vitellogenesis is indeed rhythmic, this would very probably result from rhythmicity in these regulatory mechanisms. Mating in *Drosophila* is under circadian control (Sakai and Ishida, 2001). At each mating, the male transfers a sex peptide to the female. This peptide stimulates synthesis of JH (Moshitzky et al., 1996), which in turn increases transcription of the yolk protein genes within the ovary, the uptake of these proteins into oocytes (Soller et al., 1997) and

ovulation (Ottiger et al., 2000). Mating also affects ecdysteroid levels, which affect vitellogenin synthesis by both ovary and fat body (Soller et al., 1999). Therefore, rhythmic mating activity can readily lead to rhythmic changes in both JH and ecdysteroids and consequential rhythmic stimulation of vitellogenesis.

A second unconvincing line of evidence of an 'ovarian clock' derives from the finding of *per* transcript and protein in the follicle cells of small pre-vitellogenic oocytes of *Drosophila*. However, this *per* transcript does not cycle (Hardin, 1994) and the PER protein is exclusively cytoplasmic, showing no cyclic migration to the nucleus (Liu et al., 1988; Saez and Young, 1988). This un-clocklike behaviour of *per* in the follicle cells argues against the presence of an oscillator involving *per* in the follicle cells (Hall, 1998). However, the notion of a follicle cell oscillator merits exploration partly because it is these cells that synthesise ecdysteroids in most adult insects (Hagedorn, 1985; 1989). Follicle cells are also important targets of JH (review by Wyatt and Davey, 1996). Analysis of rhythmicities in follicle cells could uncover mechanisms underlying rhythmicity in the regulation of vitellogenesis.

(b) Spermatogenesis

Rhythmicity in spermatogenesis appears not to have been examined. The absence of studies may well be related to the enduring fascination of biologists with the female system, which is usually regarded as more important to reproductive success and as well as being morphologically more impressive. Indeed, much less is known of the physiology of the male reproductive system than of the female. Nevertheless, both ecdysteroids and JHs are known to be central in regulating spermatogenesis in a complex and apparently species-specific manner (Dumser, 1980; Hagadorn, 1985; Hardie, 1995; Koeppe et al., 1985; Wyatt and Davey, 1996). Both hormones act directly on the testis where they regulate rates of cell division, often in cells at specific stages of spermatogenesis. For example, JH affects the rate of division in spermatocytes of *Bombyx mori* (Kajiura et al., 1993) and ecdysteroids stimulate mitosis in spermatogonia of *Rhodnius prolixus* (Dumser, 1980) and the advance of spermatocytes to meiotic metaphase in both *Ostrinia nubilalis* (Gelman et al., 1988) and *Manduca sexta* (Friedlander and Reynolds, 1988). These hormonal controls over spermatogenesis receive input from factors such as mating. As noted for oogenesis, rhythmic inputs to the endocrine control pathways potentially create rhythmicity in these pathways and in resulting spermatogenesis. Endogenous rhythmicities within these pathways may also exist.

(c) Ovulation and egg transport

The release of eggs into the oviducts (ovulation) and their subsequent movement through the ducts is regulated by a multiplicity of hormones. Although ovulation is followed rapidly by oviposition in many species, the two processes are controlled separately. In the case of *Rhodnius prolixus* (Davey, 1985; Nijhout, 1994), terminal vitellogenic oocytes inhibit the maturation of younger follicles via an *oostatic hormone* that is released from fine abdominal nerves near the oviducts in response to stretch. This hormone acts on the follicle cells that surround younger oocytes where it antagonises the vitellogenic action of JH. Vitellogenic ovaries secrete ecdysone which acts on the brain and causes it to release a myotropic hormone (related to FMRF-amide) that promotes contraction of the oviducts and ovulation (Kriger and Davey, 1984). This sequence of events further requires the presence of a hormone released from the spermatheca of mated

females. Thus, five hormonal factors are involved in the coordination of ovulation. Comparable, though species variable, endocrine regulatory schemes are available for *Thermobia domestica* (Thysanura), *Diploptera punctata* (Blattaria), *Locusta migratoria* (Orthoptera) and *Aedes aegypti* (Diptera) (reviewed by Nijhout, 1994). The manner by which these factors are coordinated to generate a circadian rhythm of oviposition (Section D8) has not been explored.

Following their ovulation, eggs are moved along the oviducts in preparation for oviposition. The eggs are moved along the oviducts by contractions of muscles in the oviduct walls. The factors regulating muscular contractions of the oviduct have been especially fully studied in *Locusta migratoria* (references in Donini et al., 2001). The ducts are innervated from the transverse nerves, the caudal sympathetic system and neurons locally associated with the ducts. Both the myogenic and neurally-evoked contractions of the oviduct are modulated by numerous hormones and/or neurotransmitters, including proctolin, CCAP, FMRF-related peptides, octopamine and glutamate. The complexity of the mechanisms that regulate ovulation and egg transport contrasts with the analogous mechanisms for moving sperm bundles along the vas deferens, which are said to occur without involvement of either nerves or hormones in moths (Giebultowicz, 2000), as discussed below.

(d) Sperm release and transport

Sperm, like eggs, develop within the gonad inside a membranous cyst. Cell divisions within the cyst result in numerous (commonly 256) spermatids enclosed within the cyst membrane. These cysts are liberated from the testis into short vasa efferentia which connect each testis follicle to the vas deferens. In Lepidoptera, the cyst membrane is destroyed as the sperm leave the testis, an event highly comparable to ovulation in the female. The apyrene (sterile) sperm that were in the cyst rapidly become dispersed in the ducts, whereas the eupyrene (fertile) sperm become secondarily embedded within a secretion of the vas deferens (Thibout, 1980). These secondary aggregations of germ cells are known as 'bundles'. The functional significance of these sperm associations is unknown (Szöllösi, 1982). These bundles are moved down the vas deferens by muscular contractions and may remain intact until after ejaculation. Some authors refer to both the cysts within the testis and the secondary aggregations as 'bundles', even though the two packages differ in both structure and composition. The regulation of these movements of sperm have been extensively studied in certain moths. The liberation of sperm from the testis and the subsequent translocation of bundles along the duct system is known to be under circadian control. But, in remarkable contrast to the female system, the roles of nerves and hormones are enigmatic.

Riemann and Thorson (1971) observed that sperm were visible in the vasa deferentia of adult *Ephestia (Anagasta) kueiella* only at certain times of the day. Reimann et al. (1974) found the largest numbers of sperm at the end of the photophase and that the numbers were maintained in DD, but reduced in LL. Sperm bundles remained in the upper vas deferens (UVD) (just outside the testis) for 10 to 12 hours (i.e. throughout the scotophase) and were then rapidly translocated to the seminal vesicle (SV), where they remained for 5 hours before being moved along the lower vas deferens to the duplex (where they accumulate until mating). The literature describes the release of sperm from the testis and its subsequent movement down the ducts as two component 'steps' of a single rhythm of sperm release. The rhythm was maintained in isolated abdomens and responded to phase shifts of the light cycle applied to isolated abdomens (Fig. 5.21); this showed that both photoreceptor and circadian clock were contained in the

abdomen (Thorson and Riemann, 1977). Similar rhythms of sperm movement have been found in *Pectinophora gossypiella* (LaChance et al., 1977), *Lymantria dispar* (Giebultowicz et al., 1989), *Cydia pomonella* (Giebultowicz and Brooks, 1998) and *Spodoptera littoralis* (Bebas et al., 2001).

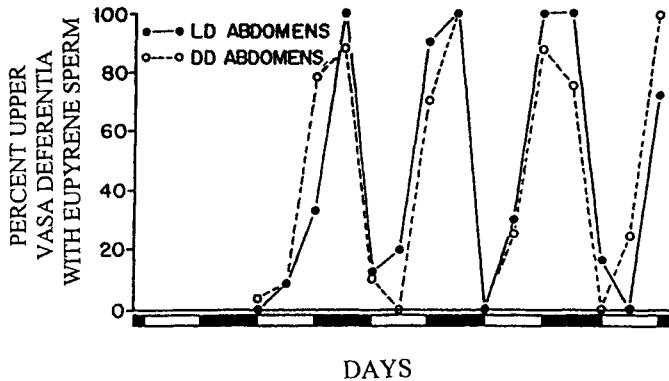


Fig. 5.21. Entrained rhythm (solid line) and free-running rhythm in DD (dashed line) of release and movement of eupyrene sperm bundles in the ducts of the reproductive system in isolated abdomens of the Mediterranean flour moth, *Anagasta kuehniella*. The rhythm is maintained if TTX is injected. Dark bars indicate scotophase. (After Thorson and Riemann, 1977).

Paralysis of animals with tetrodotoxin was without affect on the rhythm of sperm movement in isolated abdomens (Thorson and Riemann, 1977). However, a role of the nervous system was not discounted because various effects were reported of removal of the abdominal ganglia from which the reproductive tract is known to be innervated (Libby, 1961; Ruckes, 1919).

The testis-UVD-SV complex of *Lymantria dispar* maintains a rhythm of sperm movement for 2 to 3 days *in vitro* (Giebultowicz et al., 1989). By the third day, the amount of sperm in the ducts had decreased by 90 per cent. The rhythm free-ran in DD and responded to phase-shifts *in vitro* (Giebultowicz et al., 1989). Both photoreceptor and the clock regulating sperm movement are therefore within the testis-UVD-SV complex. The rhythm in *Spodoptera littoralis* also persists *in vitro* (Bebas et al., 2001), but *Ephestia kuehniella* and *Cydia pomonella* do not show rhythmicity *in vitro* (Bebas et al., 2001). The significance of these apparent species variations is not known. The testis-UVD-SV complex appears to contain both nervous and endocrine cells. The published data can be interpreted as evidence that both of these participate in regulation of rhythmicity. The testis itself is a major endocrine organ that releases ecdysteroids *in vitro* (see below).

The nerve supply was described by Riemann and Thorson (1976) and Thorson and Riemann (1977) as conspicuous in the lower vas deferens but more sparse, although not absent, in the UVD. The UVD consists of morphologically separate layers of longitudinal and circular muscle and an inner epithelium. Giebultowicz et al. (1996) reported structures resembling axons between the circular muscles and the basement membrane of the UVD epithelium. The most compelling evidence of a local nerve supply came from analysis of the complex patterns of

muscular contraction exhibited by the testis-UVD-SV complex *in vitro* (Giebultowicz et al., 1996). Several distinct patterns of contraction were seen in the UVD at different times of day, each of which required differently coordinated contractions of the circular and longitudinal muscle layers. Abrupt switches between these patterns were also seen. The complexity of patterns and abrupt switching between patterns both imply the presence of local neural regulation. By contrast, Giebultowicz et al. (1996) state that the musculature appears to receive no innervation and that the muscles autonomously undergo changes in contractile pattern. Myogenic rhythms in visceral muscle are usually achieved by electrical coupling between muscle cells (Miller, 1975), as they are in the analogous system in the female (i.e. the oviducts) (Orchard and Lange, 1988). Control of the oviducts derives from fine nerves associated with the oviducts and/or adjacent fat tissue that adheres to the oviducts during dissection; these nerves release numerous neurochemical agents (e.g. CCAP, proctolin, FMRF-related peptides, octopamine, glutamate; for details see Donini et al., 2001) that can stimulate or inhibit both the myogenic and neurally-evoked contractions of the oviducts. It is probable that local nerves around the UVD are involved in both coordination of the various patterns of contraction of the UVD described by Giebultowicz et al. (1996) and in the abrupt switching observed between these patterns. One of these patterns is responsible for mass transfer of sperm down the UVD to the SV (Giebultowicz et al., 1996). The onset of this pattern was sometimes seen after recordings had commenced *in vitro*, showing it was not driven directly from the CNS. This pattern commenced at the normal time of day for sperm transfer *in vivo* and therefore seems to be a major controlled output of the clock. Possibly the clock may control the switching between motor patterns to the UVD muscles.

The testis-UVD-SV complex also contains the largest endocrine organ of adult males, the testis. The testis is the only confirmed site of synthesis of ecdysteroids in adult male moths. It has been shown to produce ecdysteroids *in vitro* (Loeb et al., 1984; 1988) and it is also known that ecdysteroids regulate sperm release. Injection (Thorson and Riemann, 1982) or infusion (Giebultowicz et al., 1990) of 20E was found to inhibit release of sperm from the testis in a dose-dependent manner, with lower doses producing less inhibition. Giebultowicz et al. (1990) found the effectiveness of 20E injection decreased as development of the pharate adult proceeded and inferred that a decline in the ecdysteroid titre was necessary for the initiation of sperm release in the pharate adult. However, Thorson and Riemann (1982) had previously obtained similar results with 20E injections into adult moths, in which the ecdysteroid levels had already declined. They reported that the inhibition of sperm release did not appear to be due to reduced spermatogenesis because there was an accumulation of unreleased eupyrene sperm cysts at the junction of the testis and UVD in animals injected with 20E. The release of apyrene sperm was unaffected. Further, the transport of sperm down the UVD was also not affected by 20E. These differential actions of 20E on sperm release and sperm transport suggest these two processes may represent distinct rhythms. The occurrence of ecdysteroid synthesis by testis-UVD-SV complexes, even *in vitro*, is particularly important because this may be rhythmic (see Section D1). It is therefore likely that the local nerve supply, neuropeptides and ecdysteroids all interact to regulate the timing of sperm movement.

In contrast to the numerous studies of the movement of sperm along the ducts, rather little is known of the mechanism by which sperm are released from the testis into the UVD. The single layer of epithelial cells lining the UVD continues across the junction of the UVD with the testis (Riemann and Thorson, 1976; Giebultowicz et al., 1997). During the late photophase, sperm cysts can be seen traversing this epithelium and entering the UVD; the cyst membrane is broken

as they do so (Giebultowicz et al., 1997). There are several hypotheses concerning how this movement might be achieved. In the first, the epithelial monolayer is assumed to represent a barrier through which sperm cannot pass; *per* expression in the epithelial cells (described below) is suggested to regulate the rhythmic appearance of holes ('exit channels') in the epithelium which allow sperm to leave the testis (Giebultowicz et al., 1997). It is thought that sperm release is terminated a few hours later by reconstruction of the epithelium. In the second hypothesis, sperm are squeezed through the epithelium by contraction of a ring of muscle on the testis side of the epithelium (Giebultowicz et al., 1997), where no *per* expression is seen. Contractions of this muscle are described. The latter hypothesis would probably involve nervous control of the muscle. A third possibility, not discussed in the literature, is that rhythmic release of sperm might be a consequence of rhythmic spermatogenesis which could result in accumulation of sperm cysts at the membrane at a particular time each day; this possibility accommodates the effects of ecdysteroids on these processes noted above.

The location of the clock controlling sperm movement is not known. The male reproductive system of *Cydia pomonella* expresses *per* (Gvakharia et al., 2000). Relative *per* mRNA levels cycled in LD but showed 'statistically insignificant' fluctuations in DD and were 'disrupted' in LL. Both *per* mRNA and PER were localised to the wall of the UVD, reportedly to its epithelial lining. There is no direct evidence linking *per* expression to any of the overt rhythms in the reproductive system. Gvakharia et al. (2000) speculate that *per* in the epithelium may comprise a circadian mechanism involved in the gating of sperm release. It is also possible that *per* might regulate the rhythmic secretion from the epithelial cells into the lumen (Riemann and Giebultowicz, 1991; Giebultowicz et al., 1994) and the acidification of the lumen contents (Bebas et al., 2002). These secretions may participate in sperm maturation, which is completed in the UVD (Riemann and Thorson, 1976; Riemann and Giebultowicz, 1992). However, it seems unlikely that the epithelial cells could coordinate the motor patterns of muscular contraction that are involved in sperm movement.

The time during adult development at which the sperm release clock becomes operational was examined in *Lymantria dispar* by Giebultowicz and Joy (1992). Pupae were transferred from LD to DD (or given a light pulse in DD) at various times during adult development. The earliest day on which a light cue initiated rhythmicity in sperm release was pupal day 6, two days before sperm release normally commences. This is the time of peak ecdysteroids in the haemolymph (Giebultowicz et al., 1990;) and testis (Loeb et al., 1984; 1988), suggesting that the clock differentiates under ecdysteroid regulation. In an attempt to determine if 20E interacted with the clock in the reproductive system, 10-20 µg 20E were injected into animals that were maintained in LD. Appearance of sperm in the UVD was abolished for one day but then resumed, as previously found in adult *Ephestia*. It was inferred that 20E does not affect the phase of sperm release. However, it is unlikely that a phase-shift could have been produced by 20E in this experiment due to the presence of conflicting light entrainment.

The biological significance of rhythmicity in sperm release and transport are unknown. Nevertheless, this system presents valuable opportunities for study of the interactions of nerves and hormones in the regulation of a peripheral oscillator. The shortage of information regarding nervous and hormonal regulation is not compelling evidence that the clock is autonomous.

3. Chemical communication: Pheromones

(a) Sex pheromones

Sex pheromones are used as signals for sexual communication between individuals of the same species. In most Lepidopteran species, sex pheromones are released by the females, when in a sexually receptive state, and function to attract conspecific members of the opposite sex for mating. Sex pheromones play a key role in sexual attraction, activation of the mating behaviour and general physiology of reproduction and are consequently essential for species survival since they ensure reproductive success.

Sex pheromones in moths are small size, volatile chemical compounds; they are primarily aliphatic hydrocarbons, which are synthesised *de novo* from fatty acids with corresponding carbon chain lengths, positions of double bonds and stereochemistry to that of the pheromones they give rise to (Chapman, 1998; Tillman et al., 1998). There is great variety in the chemical structure of sex pheromones among species. Many species release cocktails of pheromone molecules the components of which may function in a synergistic fashion. The release of species-specific cocktails contributes to the species-specificity of the response. Synthesis of pheromones in moths occurs mainly in the pheromone gland, which is usually located beneath the intersegmental membrane between two posterior abdominal segments, usually between the 8th and 9th segments. In many lepidopteran families, the pheromone glands are bulbous extrudable sacs derived from modified epidermal cells (Bjostad et al., 1987). Usually, these glands have no reservoirs, thus the sex pheromone is released directly into the environment following synthesis. Release of sex pheromone into the environment occurs at specific times of the day (see below) and is accompanied by a specific behaviour by the female that is termed 'calling', which involves movements of the abdomen that facilitate exposure of the gland to the air and pheromone dispersal. In some species, such as *Bombyx mori*, release of pheromone is facilitated by eversion of the gland by changes in haemolymph pressure, brought about by reversal of the heart; retraction is caused by muscular action (Ichikawa and Ito, 1999). Calling behaviour has thus been associated traditionally with pheromone release.

Males respond to the female sex pheromone with orientation and upwind flight behaviour within the pheromone plume in the atmosphere. Such behaviour leads the males to the females. The male response is therefore confined to the time of day when the female releases pheromone. In some species, the rhythm of male responsiveness to pheromone is regulated by a circadian clock in the male that is independent of, but normally synchronised with, the rhythm of pheromone release by the females (see Section D4).

In some species, primarily members of the Diptera, it is the male that releases sex pheromone and the female that responds to it. In the higher flies, such as *Musca domestica* and *Drosophila melanogaster*, discrete pheromone glands are not present; rather, pheromones are synthesised by abdominal oenocytes (see Tillman et al., 1999) and transported to epidermal cells for deposition on the cuticle surface. In *Anastrepha suspensa* there is a daily rhythm of pheromone emission and calling behaviour by males (Epsky and Heath, 1993); the male haemolymph shows a daily rhythm of pheromone molecules (presumably in transport) that is synchronised with that of emission (Fig. 5.22) (Teal et al., 1999b).

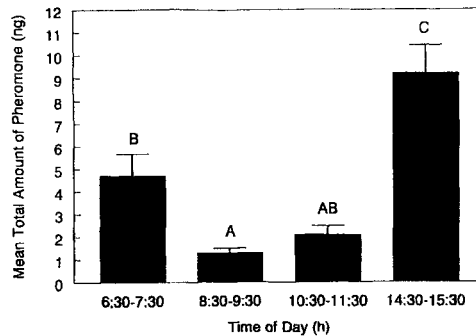


Fig. 5.22. Daily changes in the haemolymph content of sex pheromone(s) in males of the Caribbean fruit fly, *Anastrepha suspensa*. These changes correspond closely to the daily changes in pheromone release. (From Teal et al., 1999b).

(b) Circadian rhythms in pheromones in moths

One of the earlier observations in moths was that calling behaviour by the adult female, as well as responses of males to calling, occurred at specific times of day, either in the scotophase or photophase, depending on whether the moth species was nocturnal or diurnal. In the nocturnal moth *Helicoverpa* (formerly called *Heliothis*) *zea*, for example, virgin females do not call in the first scotophase after emergence; calling initiates during the second scotophase and continues rhythmically every night for several nights until mating. Mating almost completely inhibits calling behaviour in moths. The calling rhythm was shown to be under circadian control in many studies (see below). The striking periodicity of calling in female moths, implied that the associated pheromone release was also rhythmic; indeed, a large number of studies confirmed clear daily rhythms of both pheromone content of the pheromone gland as well as pheromone release by the gland. Close temporal relationships were almost invariably established between content and release (see below). In general, the temporal pattern of pheromone content of the gland is similar between different species: the pattern consists of peaks of high levels of pheromone at a specific time in the circadian cycle, alternating with deep troughs characterised by low levels or near absence of pheromone. Likewise, release of pheromone in the form of emission of pheromone plumes, also occurs at a specific and narrow window during a day either in scotophase or photophase depending again on the species. Thus, in many moths, all three rhythms - gland content, release and calling behaviour - run apparently co-phasically. For example, synchronous rhythms of calling and pheromone content of the gland have been observed in a large number of moths including *Adoxophyes* spp (Kou, 1992), *Bombyx mori*, (Sasaki et al., 1984; Kuwahara et al., 1983), *Choristoneura fumiferana* (Ramaswamy and Cardé, 1984), *Mamestra brassicae*, (Noldus and Potting, 1990; Iglesias et al., 1999) *Phthorimaea operculella* (Ono et al., 1990), *Platynota sultana* (Webster and Cardé, 1982), *Sesamia nonagrioides* (Babilis and Mazomenos, 1992) and *Spodoptera littoralis* (Dunkelblum et al., 1987). As mentioned above, when the pheromone gland is not associated with a reservoir, production of pheromone leads directly to its release. Therefore, periodic changes in the pheromone content of a gland with no storage facility ought to result from periodic changes in the production of pheromones by the gland coupled by periodic release. Indeed, Foster (2000) showed that the diurnal rhythm of pheromone content in *Epiphyas*

postvittana glands was the result of two synchronous rhythmic biochemical processes, a daily cycle of synthesis coupled with a daily cycle of release, but no daily cycle of pheromone degradation. In species where rhythmic release of pheromones results from rhythmic synthesis, the neuroendocrine regulation of the pheromone gland is likewise rhythmic (see below). However, there are cases in which pheromone production and release are not synchronous, as is the case with *Trichoplusia ni*. In this moth, pheromone is released with a circadian rhythm (Sower et al., 1970). Rhythmic changes in pheromone content of the gland result from rhythmic depletion of pheromone reserves in the gland rather than rhythmic production; instead, biosynthesis of the major component of pheromone is continuous in this moth without major daily fluctuations (Hunt and Haynes, 1990). The authors suggested that in *Trichoplusia ni* periodicity in emissions is a manifestation of an underlying periodicity of transport of pheromone to the gland surface that is correlated with pheromone release behaviour. All three phenomena, pheromone production/titre, pheromone release and calling have been shown to be under endogenous circadian control in many moth species, as described below.

In *Lymantria dispar*, the pheromone content of the gland (Tang et al., 1992; Webster and Yin, 1997), the rate of pheromone emission from the gland (Charlton and Cardé, 1982) and the calling behaviour (Giebultowicz et al., 1992; Webster and Yin, 1997) all exhibit clear, synchronous daily rhythmicities. All daily rhythms display low values at the beginning of the photophase, peak in the late photophase and decline to minimum during the scotophase. In *Lymantria*, the calling rhythm free-runs in DD with τ shorter than 24 hours (Webster and Yin, 1997). Transfer of females to LL abolishes rhythmicity in calling but does not abolish the behaviour; females in LL called continuously. Transfer from LL to DD reinstated rhythmicity. Thus, rhythmicity in calling is under circadian control. Interestingly, initiation of the calling rhythm seems not to require the presence of pheromone in the pheromone gland; newly emerged females exhibited the behaviour even though there was no pheromone in their glands. The behaviour was also evident at temperatures low enough to inhibit pheromone production (Giebultowicz et al., 1992).

Daily, parallel fluctuations in both the pheromone content of the gland (Mbata and Ramaswamy, 1990; Rafaëli and Soroker, 1989) and emissions from the pheromone gland (Pope et al., 1982) were also observed in *Heliothis virescens*; these rhythms displayed maxima in the middle to late scotophase and minima in the photophase. In a related species, *Helicoverpa armigera*, the calling rhythm was endogenous in nature since it free-ran in DD (Kou and Chow, 1987).

All five components of the pheromone blend in the *Helicoverpa assulta* showed a daily rhythm; high quantities were detected throughout the scotophase but none in the photophase (Choi et al., 1998). Calling is also rhythmic in this species and occurs synchronously with the rhythm of pheromone content of the gland (Kamimura and Tatsuki, 1993). Both pheromone and calling rhythms are controlled endogenously since they both free-run in aperiodic conditions. The pheromone rhythm free-runs in both DD and LL with a τ value similar to that in LD. The calling rhythm also free-runs in DD in parallel to the pheromone rhythm (Fig. 5.23). Interestingly, the two rhythms respond differently to LL; the calling rhythm was either abolished or became irregular, whereas the pheromone rhythm free-ran (Kamimura and Tatsuki, 1994).

In *Pseudaletia unipuncta*, both the proportion of females in a population whose pheromone glands contained pheromone and the relative pheromone content of glands of individual females fluctuated markedly during a day in LD; both parameters were close to nil at the onset of the scotophase and then peaked in the latter half of the dark, at which phase calling behaviour was also observed. All parameters decreased drastically during the

photophase. When females were transferred to DD after the first night of active calling, both calling behaviour and the rhythms of pheromone content and release showed free-running behaviour (Delisle and McNeil, 1987).

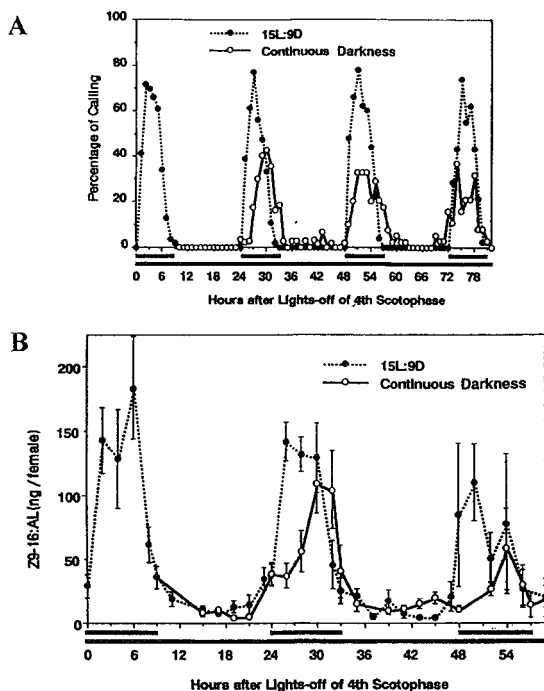


Fig. 5.23. Entrained rhythm (dotted lines) and free-running rhythms in DD (solid lines) of calling behaviour (A) and sex pheromone content of the pheromone gland (B) of females of the Oriental tobacco budworm moth, *Helicoverpa assulta*. Note that both calling and pheromone rhythms run in synchrony. (From Kamimura and Tatsuki, 1994).

The rhythm of calling in the tiger moth, *Holomelina laeae*, was found to free-run in DD but to damp out after 2 to 3 cycles in LL (Schal and Cardé, 1986). Rhythmicity of calling was restored by imposition of a single scotophase. A population of insects that was reared in DD from the second instar onwards showed no apparent rhythm of calling; but when insects were grouped according to the time of eclosion, a calling rhythm was evident. This report suggests that the clock controlling the calling rhythm may be phase-set at the time of ecdysis in DD. Alternatively, the clock may be free-running in larvae in DD, and resynchronisation of the population at ecdysis may reveal the rhythm at the population level. The calling rhythm is also circadian in *Manduca sexta*, since it free-runs in DD with a period length of 25.4 hours (Itagaki and Conner, 1986).

Temperature compensation of the calling rhythm appears to have been demonstrated in only one species. Females of *Congethes punctiferalis* were transferred to DD under various temperature conditions which were different from the temperature in LD. They all exhibited free-running rhythms with a period length closely similar to that of the entrained rhythm in LD (Kaneko, 1986).

Various affects of temperature on pheromones or calling behaviour other than compensation of the period length of the free running rhythm have been noted in a variety of moths (Baker and Cardé, 1979; Chalton and Cardé, 1982; Delisle and McNeil, 1987; Giebulowicz et al., 1992; Haynes and Birch, 1984; Webster and Cardé, 1982; Webster and Yin, 1997).

Sasaki et al. (1987) found the calling rhythm was entrained by brain photoreception in *Andevidia peponis*. Insects with both compound eyes and ocelli surgically removed responded to phase-shifts of the LD cycle. Various regions of the brain were subjected to localised illumination with 100 µm fibre optic light guides. Illumination of most brain areas, including the optic lobes, was ineffective; phase-shifts were induced only by illumination of the medio-dorsal protocerebrum, in the vicinity of the medial neurosecretory cells.

Collectively, the above studies established that the rhythms of pheromone gland content, pheromone release and calling are all under circadian control.

(c) Rhythmicity in neuroendocrine mechanisms that control pheromone production

Two hormones are known so far to play a crucial role in pheromone production in moths, the neuropeptide *pheromone biosynthesis activating neuropeptide* (PBAN) and the develop-mental hormone, *juvenile hormone* (JH).

Riddiford and Williams (1971) proposed that the head was essential for the production of pheromones. A breakthrough in the study of physiological mechanisms in the regulation of pheromone production and consequently the understanding of circadian control of pheromone production, was the discovery in several moth species of a factor in the brain-retrocerebral-suboesophageal complex (brain complex) with pheromonotropic activity (Ma and Roelofs 1995; Martinez and Camps, 1988; Rafaeli and Soroker, 1989; Raina and Klun, 1984). For example, both pheromone production and calling behaviour were abolished by decapitation. However, injection of brain complex extract into decapitated females re-instated pheromone production. Likewise, injection of extracts from brain complexes into intact females at circadian times of no pheromone production, or into decapitated females, promptly induced both the production of pheromone and calling behaviour. This brain-complex factor was eventually identified as a small neuropeptide, pheromone biosynthesis activating neuropeptide (PBAN). PBANs have been identified and sequenced in several moths, including *Bombyx mori* (Kitamura et al., 1989) *Helicoverpa zea* (Raina et al., 1989; Choi et al., 1998), *Lymantria dispar* (Masler et al., 1994) and deduced from cDNA sequences in *Agrotis ipsilon* (Duportets et al., 1998), *Helicoverpa assulta* (Choi et al., 1998) and *Mamestra brassicae* (Jacquin-Joly et al., 1998). PBAN is synthesised as a preprohormone that is post-translationally trimmed to several peptides with PBAN activity including a peptide that bears striking similarities to the diapause hormone of *Bombyx mori* (Sato et al., 1993) and peptide(s) involved in cuticular melanisation and coloration in *Bombyx mori* and *Spodoptera littoralis* (Matsumoto et al., 1990; Altstein et al., 1996). It is now generally accepted that pheromone biosynthesis in moths is regulated primarily by PBAN, although other means of control (neural control of the pheromone gland) appear also to be involved (see below).

In several moths, studies on the biological activity of PBAN, immunocytochemical studies and studies of expression of the PBAN gene in various tissues have identified small groups of nerve cells in the suboesophageal ganglia (SOG) as the primary source of PBAN (Ma and Roelofs, 1995; Ma et al., 1998; Ma et al., 2000; Choi et al., 1998b). The derivation of PBAN and diapause hormone (DH) from a common preprohormone in cells of the SOG implies that neural regulation pathways, such as those for light input, may be shared by these

neuropeptides. Mapping of the axonal pathways from the PBAN-positive cells in SOG revealed connections to the corpora cardiaca (CC) suggestive of transport of material from the SOG cells to the CC in *Bombyx mori* (Ichikawa et al., 1995). Assay of the PBAN content of these organs in *Helicoverpa* spp showed that the PBAN content of the brain and SOG was high throughout the photophase and declined during the scotophase; by contrast, PBAN accumulated in the CC as it was depleted from the brain-SOG (Rafaeli et al., 1991; Rafaeli, 1994). Similar cycling of levels of biological PBAN activity in these organs are reported by Raina and Menn (1987). Collectively, these results are consistent with continuous synthesis of PBAN in the SOG and its transport to the CC in time for rhythmic release during the scotophase (Rafaeli, 1994). Further, in *Ostrinia nubilalis*, removal of the retrocerebral complex significantly reduced the production of pheromone by the pheromone gland; furthermore, CC immunostained intensely for PBAN and exhibited strong PBAN biological activity (Ma and Roelofs, 1995). These findings imply that PBAN is likely to be released into the haemolymph at the CC.

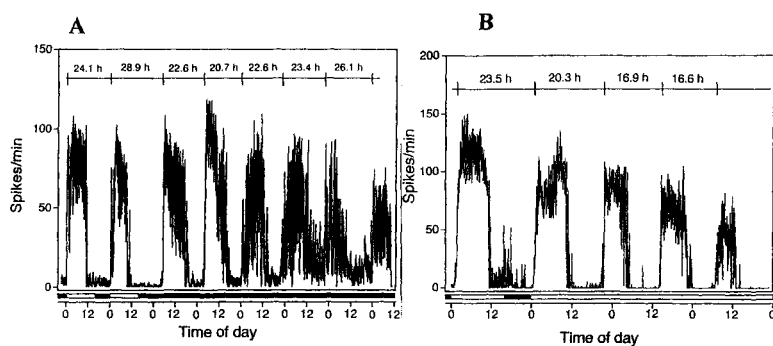


Fig. 5.24. Circadian rhythm of electrical activity in the nerve from the suboesophageal ganglion to the corpus cardiacum of female silkworm, *Bombyx mori*. The nerve carries the axons of pheromone biosynthesis activating neuropeptide (PBAN) cells and electrical activity is an indicator of PBAN release. The rhythm free-runs in both DD (A) and LL (B), with a period length shorter than 24 hours. (From Tawata and Ichikawa, 2001).

Extracellular recording of the electrical action potentials of the PBAN-positive cells in SOG in *Bombyx mori* revealed a clear daily rhythm of bursting firing with two components; the peaks of the major component occurred during the scotophase (Ichikawa, 1998). The rhythm of the major peaks is synchronous with rhythmic changes in pheromone content of the gland and abdominal movements characteristic of calling behaviour. The daily rhythm of electrical activity in the PBAN cells free-ran in both DD and dim LL indicating that the firing rhythm is under circadian control (Fig. 5.24A, B). This finding indicates that PBAN release is rhythmic and under circadian control (Tawata and Ichikawa, 2001) (see also below). PBAN stimulated the production of pheromone by *in vitro* preparations of pheromone gland (Rafaeli and Soroker, 1989; Rafaeli, 1994; Jurenka et al., 1991), suggesting that the peptide may act humorally *in vivo*. Rafaeli and Gileadi (1997) identified a cell membrane protein on pheromone glands that binds specifically to PBAN, further supporting the notion that PBAN acts directly on the pheromone gland. Indeed, PBAN appears to act on pheromone glands *in vitro* assays via a Ca^{++} /calmodulin-dependent adenylate cyclase pathway system (review by Ramaswamy et al., 1994). Evidence that PBAN functions as a blood-borne hormone was obtained from bioassays and immunobinding studies that identified and quantified PBAN-like

activity in the haemolymph. Ramaswamy et al. (1995) detected PBAN-like activity in the scotophase haemolymph of females of *Heliothis zea*, coinciding with the time of peak pheromone production. Further, the authors isolated a peptide fraction from the haemolymph of *Heliothis* that stimulated pheromone production in isolated abdomens of photophase females, at a time when pheromone production would not otherwise occur. The level of this PBAN-like peptide factor fluctuated daily in the haemolymph; it was present in scotophase animals but absent in photophase animals. PBAN-like activity was also detected in the haemolymph of females of *Mamestra brassicae* (Iglesias et al., 1999). The authors measured the levels of PBAN-immunoreactivity throughout the scotophase as well as 2 hours before and 1 hour after scotophase onset. They identified a large increase of PBAN-immunoreactivity in the middle of the scotophase flanked by periods of very low levels, a pattern of fluctuations that suggested daily cycling of PBAN-like material in the haemolymph (Fig. 5.25A). It is important to note that this 'cycling' of PBAN-like immunoreactivity in the haemolymph occurred synchronously with both the rhythm of calling behaviour and the rhythm of pheromone production by the pheromone gland (Fig. 5.25B) (Iglesias et al., 1999). It is concluded that PBAN levels in the haemolymph show daily rhythmic fluctuations in several moths. Collectively, all the above suggest that the daily rhythmicity of PBAN in the haemolymph results from rhythmic daily release of PBAN from brain complexes.

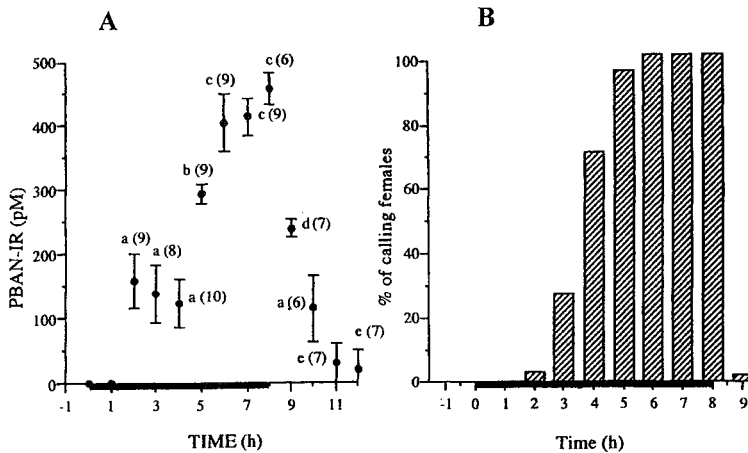


Fig. 5.25. Daily changes in pheromone biosynthesis activating peptide-like material (PBAN-like) in the haemolymph (A) and calling behaviour (B) of female cabbage armyworm, *Mamestra brassicae*. Note that the two rhythms are synchronous. For details, see text. (From Iglesias et al., 1999).

Decapitation of young, non-calling females of *Ostrinia nubilalis* two hours before the second scotophase after emergence to the adult, led to rapid decline of the pheromone level in the gland to undetectable level within 24 hours (Ma and Roelofs, 1995). Injection of SOG extract from normal scotophase females or synthetic PBAN into these decapitated females led to rapid resumption of pheromone production within 3 hours after injection to levels equivalent to that of normal scotophase females. The same degree of pheromone stimulation by injection was obtained, regardless of whether the decapitated animals were kept in dark or light. In other words, though whole animals, raised in a light cycle, display peaks of pheromone production each scotophase, in decapitated animals which had lost their pheromone rhythm, production

could be initiated at times other than scotophase, i.e. photophase. Similar observations were made in *Spodoptera littoralis* (Martinez and Camps, 1988; Rafaeli and Soroker, 1989), in *Heliothis armigera* (Rafaeli and Soroker, 1989) and in *Helicoverpa assulta* (Choi et al., 1998). All the above strongly suggest that the rhythm of pheromone production by the pheromone gland requires daily regulation by either PBAN and/or neural factors. The daily rhythm of pheromone production by the pheromone gland is therefore driven from within the nervous system.

The above picture of PBAN acting as a humoral factor is complicated by several reports that PBAN is distributed widely in the nervous system and that the pheromone gland in some species receives direct innervation. PBAN immunoreactivity has been found in cells of the brain, thoracic and abdominal ganglia of several moths (Rafaeli et al., 1991; Ma et al., 1998; Ma and Roelofs, 1995). Expression of the gene for PBAN is also seen in these diverse locations in *Helicoverpa zea* (Ma et al., 1998). PBAN levels were found to cycle daily in these locations, but the cycles were out of phase with each other in the various locations in the central nervous system (Rafaeli, 1994). In *Heliothis zea*, the pheromone gland receives innervation from the terminal abdominal ganglion (TAG) (Teal et al., 1989). These findings are consistent with the view that PBAN may be synthesised in the TAG and delivered to the pheromone gland through neural connections. Octopamine also stimulates the gland (Christensen et al., 1991; see also below). The PBAN cells of the SOG have processes that enter the ventral nerve cord (Kingan et al., 1990), but their relationship to PBAN in the TAG is unclear. It remains therefore possible that PBAN may be released from sites other than the CC, in at least some species.

The gene for PBAN is expressed in males as well as females (Rafaeli et al., 1993; Choi et al., 1998), and putative receptors for PBAN (identified by photoaffinity labelling) are reported in thoracic muscle and within the central nervous system (Elliot et al., 1997). Novel functions and sites of action of PBAN are implied other than regulation of the pheromone gland.

In several moths, such *Pseudaletia unipuncta* (Cusson and McNeil, 1989), *Agrotis ipsilon* (Picimbon et al., 1995) and *Helicoverpa armigera* (Fan et al., 1999), allatectomy or decapitation of newly emerged females irreversibly inhibited production of pheromone; production was restored by injections of corpus allatum extracts or of JH. These findings suggested that JH was essential to the regulation of sex pheromone production in these moths. Possible routes by which JH might regulate pheromone production include direct control of production and/or release of PBAN by the brain-SOG complex (Picimbon et al., 1995) or priming the action of PBAN on the pheromone gland (Fan et al., 1999). The role of JH in the expression of rhythmic production of sex pheromone in moths is discussed in Section D1.

The endocrine role of PBAN in the daily production of pheromones, described above, is not universally accepted. Several lines of evidence have indicated that pheromone production in moths may be regulated by nerves to the pheromone gland in addition to PBAN. In two *Heliothis* species (Christensen et al., 1991; Teal et al., 1999) and *Manduca sexta* (Christensen et al., 1992) the pheromone gland is innervated from the TAG, whereas in *Ostrinia nubilalis* it is not innervated (Ma and Roelofs, 1995). In species where the pheromone gland is innervated, the ventral nerve cord seems to play an important role in pheromone production. In *Heliothis zea* and *H. virescens*, for example, pheromone production is inhibited by transection of the ventral nerve cord; even so, injection of either brain-SOG extract or PBAN into intact females, during the photophase (a circadian time when these moths do not normally produce pheromone) does stimulate pheromone production (Teal et al., 1989; Christensen et al., 1991; 1992; 1994). Electrical stimulation of the connectives anterior to TAG also induced pheromone production in intact females during the photophase (Christensen et al., 1991; 1992).

Pheromone production was induced by injection of octopamine into either intact females during the photophase or isolated abdomens with transected nerve connectives from animals at the onset of the scotophase (Christensen et al., 1991; 1992). The levels of octopamine in the TAG are high just prior to scotophase but then drop precipitously after the onset of scotophase. At this time, there is an increase in octopamine levels in the pheromone gland, suggesting transport of octopamine from the TAG to the pheromone gland; octopamine levels increase in the pheromone gland in parallel to pheromone emission and female calling (Christensen et al., 1992). These experiments and others suggested that pheromone production by the gland is affected by octopaminergic efferent nerves and that PBAN may act on the TAG rather than on the gland itself. Paradoxically, Jurenka et al. (1991) and Ramaswamy et al. (1995) failed to reproduce the findings of Christensen et al. (1991; 1992) using the same experimental protocol in the same moth, *Heliothis zea*. The controversy continues because more recently Rafaeli et al. (1997) proposed that the role of octopamine was to inhibit (rather than stimulate) pheromone production in the related species *Helicoverpa armigera*. It was proposed that octopamine participates in regulation of the daily rhythm of pheromone production by suppressing production during the photophase, probably by inhibition of the pheromonotropic action of PBAN. Injection of PBAN into decapitated females induced sustained increase of pheromone production, whereas injection of PBAN into intact females in photophase (normally low or no pheromone production) induced a transient increase in pheromone production. However, injection of PBAN and the adrenergic agonist, clonidine, into intact females inhibited this transient increase. The inhibitory effect of clonidine was not reversed by adrenergic antagonists unless the head was also removed. It was concluded that the pheromone gland receives both stimulatory (PBAN) and inhibitory (octopamine) inputs that interact at the level of receptors in the gland. These findings suggest that the daily regulation of pheromone production involves rhythmicity in both the peptide and amine pathways. The sources of these rhythmicities are evidently within the nervous system, but are otherwise not localised.

4. Circadian rhythms in olfactory responses

A daily periodicity in the response to odorous molecules has been reported in many insects. In several instances it is clear that this daily rhythm is under circadian control. These timed responses to odorants facilitate detection of predators, food and potential mates. However, the functional significance of rhythmicity in the olfactory system is most apparent in the response to sex pheromones. The daily rhythm of release of sex pheromones (see Section D3b) is synchronised with a daily rhythm of responsiveness in various insects, including moths (Castroville and Cardé, 1979), cockroaches (Liang and Schal, 1990; Zhukovskaya, 1995) and flies (Pivnick, 1993; Sybchev et al., 1986). Such synchrony ensures optimal detection of the signal from the other sex, but most important, the presence of coincident rhythms of pheromone release by one sex and responsiveness by the other is a powerful mechanism for reproductive isolation.

Early observations with the moth *Trichoplusia ni* showed that the daily rhythm of behavioural responsiveness by males (upwind orientation and flight) anticipated the daily peaks of female calling behaviour (Storey, 1966). Circadian control of the rhythm of male responsiveness has been shown by transferring males to DD and exposing them to the female pheromone, either continuously or in intermittent pulses. The behavioural response of the males varied with circadian periodicity (Fig. 5.26) (Castroville and Cardé, 1979; Liang and Schal, 1990; Linn et al., 1992; 1996).

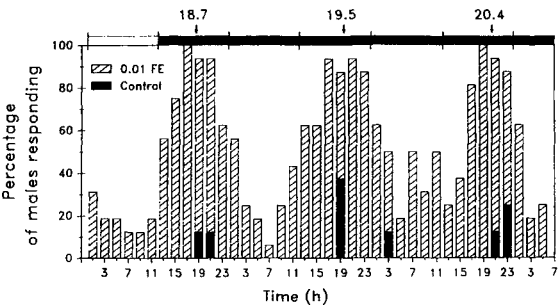


Fig. 5.26. Circadian rhythm of responsiveness of male brown-banded cockroach, *Supella longipalpa* (F.) to female sex pheromone. The rhythm free-runs in DD (dark horizontal bar on top) and shows high responsiveness each subjective scotophase and low responsiveness each subjective photophase (τ about 25 hours). Experimental males were exposed every 2 hours to female sex pheromone (FE, female equivalent) (hatched vertical bars), while control males were exposed to hexane (dark vertical bars). (From Liang and Schal, 1990).

Such circadian changes in responsiveness could, in theory, derive either from circadian changes in olfactory sensitivity in sense organs of the antennae or from circadian changes in the central processing of olfactory input. There is evidence that both mechanisms may occur.

Isolated antennae of *Drosophila melanogaster* exhibited rhythmic expression of the *per* gene, that showed both light-entrainability and free-running in DD *in vitro* (Plautz et al., 1997). The electroantennogram (EAG) response to pulses of odorants, such as ethyl acetate, revealed a circadian rhythm of olfactory responses that free-ran in DD and damped in LL (Krishnan et al., 1999) (see Chapter 4). Experiments with a *per* transgenic line (Krishnan et al., 1999) and with *cry* mutants (Krishnan et al., 2001) confirmed that the antennae of *Drosophila* possess a circadian clock that drives circadian olfactory responses (Fig. 5.27). Responses to pheromones are probably likewise regulated by the antennal clock, since pheromone receptors are located in the antennae (Clyne et al., 1997).

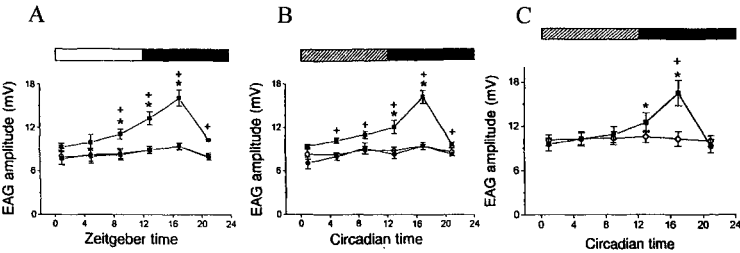


Fig. 5.27. Entrained rhythm (A) of olfactory responses of wild type *Drosophila melanogaster* to odorants (filled squares) which free-runs in DD (B; second day in DD). *per*⁰ (open circles) and *tim*⁰ (filled circles) flies show no rhythmicity. A *per* transgenic fly (*per* 7.2.2), which lacks peripheral *per* oscillators, exhibits no rhythmicity in olfactory responses (C, open circles) when kept in DD for two days. In contrast, wild type flies exhibit rhythmicity (C, squares). See text for details. (From Krishnan et al., 1999).

Octopamine influences various features of the response of male *Trichoplusia ni* to female sex pheromones, including affects on the circadian rhythm of responsiveness (Linn et al., 1996; Linn, 1997). Other reported affects of octopamine include increased sensitivity to

pheromone, improved blend discrimination, improved orientation towards the pheromone source and increased locomotor activity (Linn and Roelofs, 1986; 1992; Linn et al., 1992; 1996; Linn, 1997). The complexity and variety of affects suggests that at least some of these responses involve actions of octopamine on the central nervous system. This possibility is supported by the finding that octopamine levels in brain vary during a day in synchrony with the rhythm of pheromone responsiveness (Linn et al., 1996). These authors suggest that the rhythm of pheromone responsiveness arises centrally, perhaps driven by the locomotor clock. The role of octopamine is therefore envisaged as a modulator of outputs that are downstream of the locomotor clock. This model is reminiscent of the modulation of phonotactic behaviour of female crickets (see below).

Independently of any central action of octopamine, there is clear evidence that octopamine modulates the sensitivity of the antennal sensory neurons that detect female sex pheromone in *Antheraea polyphemus* (Pophof, 2000). Octopamine increased the action potential frequency generated by receptor neurons in response to a broad range of pheromone concentrations. Action potential frequency was reduced by the octopamine antagonist epinastine. Octopamine receptors have been cloned from antennae of *Bombyx mori* and *Heliothis virescens* and localised by *in situ* hybridisation to the base of the olfactory hairs (Nickisch-Roseneck et al., 1996). The modulatory action of octopamine appears to occur at the level of action potential generation in antennal receptor neurons. In *Manduca sexta*, octopamine seems to act by modulation of the accessory cells of the sensillum rather than on the receptor neurons themselves (Dolzer et al., 2001). In both cases, this peripheral modulation of sensitivity to pheromone by octopamine could explain a number of the more indirect findings noted above in other moth species. The neural pathway for processing olfactory input involves the antennal lobes of the brain, from whence projection neurons travel to the lateral protocerebrum. Disruption of these projection neurons in transgenic *Drosophila* results in defects in both olfactory responses and male courtship behaviour (Heimbeck et al., 2001). Therefore, there exists a neural pathway connecting olfactory input with rhythmic behavioural outputs. However, it has not yet been demonstrated that the modulation of antennal responses by octopamine contributes to the circadian rhythm of olfactory responses or whether an 'antennal clock' is found in moths.

All of these findings are consistent with a model in which an antennal olfactory clock is modulated by octopamine and its output provides an input to the central locomotor clock; the outputs of the latter would then drive rhythmic behaviours including upwind flight, courtship and mating. The central neural pathways to these rhythmic behaviours would be modulated by JH (as discussed in Section D1).

5. *Photic communication: Bioluminescence*

Some insects locate a mate by means of light signals. Such photic communication takes the form of the emission of a stereotyped pattern of flashes of light by one sex to advertise their presence to the other. The other sex frequently responds with a different, but equally stereotyped pattern of flashes of light. The advertiser then flies towards the respondent and mating follows. In some species, such as *Lampyrus*, the female is sedentary and attracts the male (which is not bioluminescent) by light emission. In other species, such as *Photuris* and *Photinus*, the male advertises during flight by emitting flashes of light in a species-specific pattern; the female replies with timed flashes. If there is no reply, the male will repeat its pattern of flashes for long periods of time. The rigidity of flash duration and inter-flash interval have made flash patterns useful as taxonomic features (Buck, 1988). Two central

generalisations are implicit in these observations. First, flashing must occur with a daily rhythm since photic communication cannot occur in daylight. Second, the repetition of a stereotyped pattern of flashing during the night suggests an ultradian component to the flashing rhythm. This section addresses both the circadian and ultradian rhythms of flashing and illustrates that the two rhythms are inter-related, both in terms of their formal properties and their physiological control.

Most bioluminescent insects belong to the Coleoptera, among which the fireflies (Lampyridae) are both the best known and most thoroughly studied. Light may be emitted by either the male or female or by both sexes, depending on species. In a few species, larval stages are also bioluminescent; in larvae, light is emitted in a prolonged glow rather than patterned flashes and functions mainly in defence or prey location (Viviani and Bechara, 1997). In adults, light is produced by paired organs known as lanterns which are located on the abdomen, beneath a patch of transparent cuticle. The lantern comprises cylinders of photocytes arranged at right angles to the overlying cuticle, usually surrounding a central trachea that branches into tracheoles that penetrate invaginations of the photocyte cell membranes (Smith, 1963). Photocytes are modified fat body cells. Light production within the photocytes results from oxidation of luciferin in the presence of luciferase, ATP and oxygen. These reactions occur within organelles known as peroxisomes. Species differences in the luciferase result in light emission at different wavelengths (McElroy and Deluca, 1985). Individual flashes of light are brief, lasting only a few milliseconds. Flashes are emitted in a precise pattern, involving several flashes at precise intervals over a precise period of time (commonly lasting about 3 to 10 seconds). A flash signal consists of a species-specific pattern of flashes which is determined by the total number of flashes and the length of the inter-flash interval (Case, 1984; Wilson and Hastings, 1998).

Light production by the lanterns is regulated by the central nervous system. In *Photuris versicolor*, the lanterns are innervated by a small number of dorsal unpaired medial (DUM) neurons whose somata reside in the last two abdominal ganglia. Each burst of electrical activity arriving at the lantern leads to emission of a flash of light (Christensen and Carlson, 1981). DUM neurons have symmetrical left and right neurites providing a pathway for synchronisation of flashing in left and right sides. The nervous system appears to contain a pattern generator that encodes the species-specific flash pattern. The DUM neurons that innervate the lantern do not terminate on the photocytes themselves, but on tracheolar cells surrounding the terminal branch points of the tracheal air supply (Ghiradella, 1977). Exposure of a lantern to air during dissection causes it to glow spontaneously. These observations led to examination of the role of regulated oxygen access to the photocytes in the control of flashing. When spontaneously flashing males of intact *Photinus* sp. were exposed to oxygen gas instead of air, the lantern was caused to glow continuously; when the oxygen was replaced with air, the lantern recommenced flashing (Timmins et al., 2001). Similarly, fireflies from the daily photophase (i.e. not flashing) responded to oxygen by commencing to glow. Analysis of electrically induced flashing in various gas compositions led to the conclusion that flashing appeared to be regulated by a gating of oxygen access to the photocytes. According to this model, a burst of action potentials in the DUM neurons would release octopamine at the tracheal end cells and cause transient water resorption from the tracheole, thereby allowing air in the tracheole access to the photocyte. It was further found in *Photuris* sp. (Trimmer et al., 2001) that nitric oxide synthase is found in tracheolar cells and/or the lateral margin of the photocytes where mitochondria are aggregated. It was proposed that this enzyme is activated by octopamine, and that the nitric oxide resulting could induce a transient inhibition of mitochondrial respiration; this would serve to transiently increase the availability of oxygen to

the peroxisomes in the centre of the photocytes. In these fireflies it seems likely that the nervous system regulates the access of oxygen to the peroxisomes. Debates continue in the literature over whether or not these processes can operate with the speed required to produce the rapid kinetics of a flash and of bunches of flashes that the lantern emits (Wilson and Hastings, 1998; Timmins et al., 2001). Interestingly, the emission of light from the peroxisomes could play a role in terminating the flash, because it is known that the nitric oxide-mediated inhibition of cytochrome c oxidase is reversed by light. In larval lanterns the photocytes are directly innervated and therefore the model of gated oxygen access through the tracheoles would not apply.

In a light:dark cycle, fireflies flash only during the evening and early scotophase. This rhythm was shown to be under circadian control by Buck (1937) in *Photinus pyralis*. In dim LL, flashing occurred for a discrete period at intervals of about 24 hours. Transfer from dark to dim light induced periodic flashing provided that the fireflies were held in dark for 24, 48, 72 or 96 hours, but not if they were held in dark for 12, 36, 60 or 84 hours. Since the free-running period of the rhythm is close to 24 hours, this experiment suggests that flashing can be induced only at certain phases of an on-going oscillation, but not at points 180° out of phase. Another study in the glow worm *Lampyris noctiluca* showed that the daily rhythm of glowing is also governed by a circadian mechanism (Dreisig, 1975, 1976, 1978). In this species, the bioluminescence rhythm is inhibited by light intensities above about 10 lux. Below this value the rhythm persists in LL with a period between 19 to 23 hours which can be phase-shifted by both light and temperature steps.

In *Photuris versicolor*, the daily peaks of flashing coincide with peak responsiveness of the compound eyes to light flashes (Fig. 5.28) (Lall, 1993). Restrained animals were kept in darkness and the electroretinogram (ERG) recorded continuously for up to 52 hours. A standardised flash of light was administered every few minutes and the amplitude of the ERG response was recorded. The ERG amplitude was low throughout the photophase, but increased about six-fold during the normal time of flashing activity. Thus, there is a rhythm of visual responsiveness (potential sensitivity) that is synchronised with the rhythm of flashing. Such synchrony would strengthen the efficacy of photic communication. In turn, flashing occurs during flight and thus must be synchronised with activity. It is therefore possible that these three rhythmic phenomena, bioluminescence, visual responsiveness and activity may all be regulated by a common circadian clock.

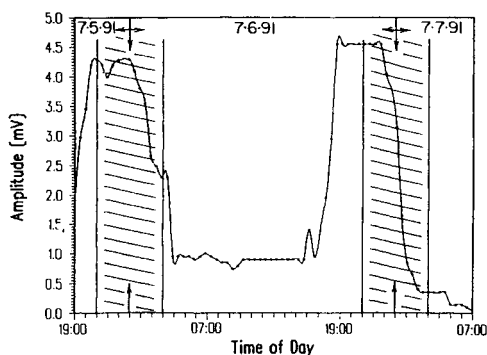


Fig. 5.28. Electroretinogram of the firefly, *Photuris versicolor*, elicited by flashes of light at constant intervals in DD. Light flashes elicit responses only at specific times of the day (solid line) that coincide with the time of daily flashing activity of fireflies *in vivo* (shaded area). See text for details. (From Lall, 1993).

The rhythmic repetition of the species-specific flashing pattern constitutes an ultradian rhythm with a period length of a few seconds. This rhythm possesses numerous properties that are analogous to those of circadian rhythms. In *Photinus pyralis*, the flashing of two males which are less than a metre apart becomes synchronised. Synchrony of flashing spreads to adjacent fireflies as the population flash pattern becomes brighter. In roving (solitary) fireflies, this synchronous flashing persists for only a few cycles, after which the population disbands (Buck, 1988). However, several species of fireflies from tropical Southeast Asia maintain synchronous flashing for a large portion of the evening and early night (Buck 1938, 1988). Buck and Buck (1968) found that the interval between the earliest and latest individual flashes in a communal flash was much shorter than the delay between electrical stimulation of the lantern and light emission. Therefore, during a mass flash, fireflies cannot be responding to each other directly. It was proposed that each firefly adjusted its period length according to whether it had flashed earlier or later than the previous average mass flash. In other words, each firefly appears able to measure time and to use this information to adjust the period length of an endogenous oscillator. Isolated fireflies exhibit a stable free-running ultradian rhythm of flashing indicating its endogenous nature. The flashing rhythm can be phase-shifted by a single, short light signal, such as from a flashlight. Such phase-shifts occur *via* transient cycles and may be advances or delays, depending on the time during the interflash interval at which the tester flash is given (Hanson et al., 1971; Buck et al., 1981a, b). Phase-response curves (see Chapters 2 and 3) have been obtained for three species of fireflies (Fig. 5.29) (Hanson, 1978). The synchronous flashing of fireflies can be modelled as a population of interacting photosensitive oscillators in which synchrony is achieved by mutual entrainment (Edmunds, 1988) (see also Chapter 7).

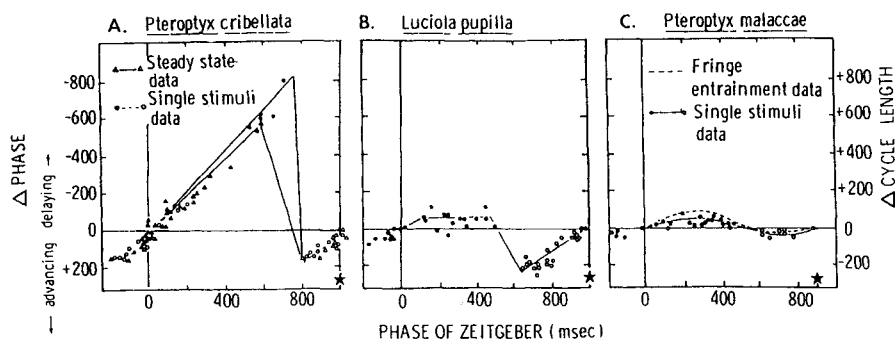


Fig. 5.29. Phase response curves (PRCs) for the circadian rhythm of flashing in three different species of synchronously flashing fireflies. The PRCs show species-specific differences of the effect of light applied at different times on the ultradian flashing cycle. *Pteroptyx cribellata* (A) has a 'strong' Type 0 PRC, whereas *Luciola pupilla* (B) and *Pteroptyx malacca* (C) both have 'weak' Type 1 PRCs. (From Hanson, 1982).

Flashing can be either enhanced or suppressed by appropriate photic or electrical stimulation of the compound eye and each flash from the lantern is preceded by a volley of action potentials in the nerve cord (Case and Buck, 1963; Case and Trinkle, 1968). Therefore, the compound eyes appear to be the receptors for entrainment and the motor programme that generates the flash pattern resides in the brain. Ablation and local nerve stimulation experiments indicated that the oscillator controlling flashing was located in the brain. A similar

conclusion was reached by Bagnoli et al. (1976) for *Luciola lusitanica*. In this species, the ultradian flashing oscillator was localised to the optic lobes. As noted above, there is also a circadian rhythm of flashing that is synchronised with flight activity and hence probably regulated by a circadian oscillator in the optic lobe (see Chapter 8). There is therefore some suggestion that ultradian and circadian oscillators may reside in the same region of the brain (Buck, 1988). This association of ultradian and circadian rhythms recalls the association in *Drosophila* between the ultradian courtship song and flight activity (see Section D6 below and Chapter 4). In *Drosophila*, both rhythms require *per* expression, but genetic mosaic flies revealed that the ultradian rhythm involved *per* expression in thoracic ganglia rather than the brain (Konopka et al., 1996). Together, these observations indicate that ultradian and circadian rhythms appear to share functional, and possibly (but not necessarily) anatomical elements as well.

6. Acoustic communication: Stridulation and courtship song

Many insects communicate using sounds. Some sounds may be adventitious consequences of the insect's activities, such as the sound of wing vibration during flight. Others are produced by specialised structures and are of special functional significance. Of the latter, rhythmicity is most frequently found in the sounds produced as part of the processes of mate location and courtship. In these instances, the rhythmicity in sound production is coordinated with numerous other aspects of reproduction, many of which are also rhythmic. The underlying control of all these events is achieved by the reproductive hormones. As noted in other sections, little is known of the mechanisms by which these hormones regulate these events, with the conspicuous exception of egg development (Sections D2a and c).

A wide variety of structures is employed in sound production in different species. In stridulation, air vibrations are created by movement of a cuticular ridge (the 'scraper') on one part of the body over a toothed ridge (the 'file') on another. The detailed design of these structures varies greatly with species, resulting in species-specific sound. However, it must be noted that an individual insect can generate several different kinds of sounds with the same apparatus. These are produced in different situations. For example, a calling song may be produced to attract the opposite sex (in synchrony with pheromone release, where this occurs; Section D3), which is modified into a courtship song when the sexes are close together. There may also be an aggression song that is produced by one male in the presence of another. These facts imply the presence of several distinct pathways in one insect for neural control of the sound-producing apparatus.

The control of stridulation is best understood in Orthoptera such as crickets (Kutsch and Huber, 1989) and grasshoppers (Hedwig, 1995; Heinrich and Elsner, 1997). Central pattern generators for the various sound patterns are located in thoracic ganglia corresponding to the segment where the muscles that move the sound-producing appendages are located (Fig. 5.30). The basic pattern is produced by the pattern generators, but is modulated by sensory feedback from proprioceptors in the stridulation apparatus and from auditory input resulting from the insect's own song. Command neurons in the protocerebrum initiate the various song patterns by activating specific pattern generators (Fig. 5.30). Execution of particular song patterns is regulated by the balance between excitatory and inhibitory activity in the command neurons. The pattern generators appear to send information forwards to the suboesophageal ganglion, where it may be employed in coordination of stridulation with other activities (Lins-Elsner, 1995). Stridulatory calling and courtship songs are under circadian control in

numerous species (see Chapter 2). The protocerebral command neurons are an obvious possible site for circadian neural input into the above control pathway.

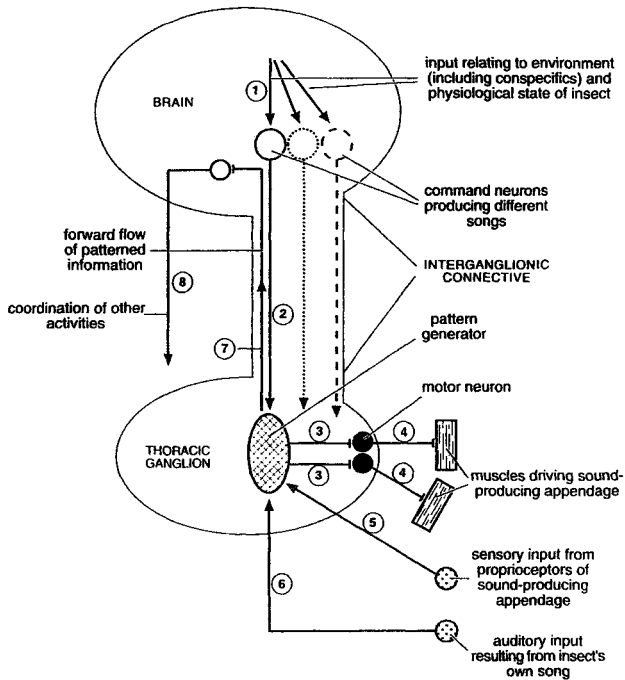


Fig. 5.30. Neuronal pathways of sound production in crickets and grasshoppers. All steps are not necessarily demonstrated in any one species. Numbers show the sequence of events. Circadian input likely occurs at '1'. (From Chapman, 1998).

The location of the oscillator controlling rhythmic stridulation has been extensively studied in the cricket, *Teleogryllus commodus*. Stridulation commences about 2 hours after the onset of the scotophase and free-runs in both DD ($\tau = 23$ to 24 hours) and LL ($\tau = 25.4$ hours) (Sokolove and Loher, 1975). Removal of the eyes or severance of the connection of the optic lobes with the eyes results in a free-running rhythm of stridulation in either LD or LL, but with the period length characteristic of DD (Loher, 1972; Sokolove and Loher, 1975). These early experiments were consistent with the view that stridulation is regulated by the same circadian oscillator that regulates locomotion. A number of subsequent experiments (Weidenmann, 1983; Weidenmann and Loher, 1984; Weidenmann et al., 1988) demonstrated that the two rhythms could be uncoupled, but the oscillators controlling both rhythms were in the optic lobes. It was concluded that each optic lobe contains two circadian oscillators (one for each rhythm) that are normally coupled together.

In crickets and grasshoppers, the stridulatory calling song pattern switches to the courtship song when the sexes are in visual contact. The courtship song is often less loud and composed of higher frequency sounds. Other insects produce a courtship song but no calling song. This is the case in *Drosophila*, where the courtship song is produced by wing vibrations. The courtship song is detected as movements of particles by the antennae (Cook, 1973).

Rhythmicity in courtship appears to be coupled to the reproductively inter-related rhythms of pheromone release and mating (Sakai and Ishida, 2001).

The courtship song of *Drosophila* consists of a series of pulses, the interval between which (inter-pulse interval) is not constant, but oscillates with a periodicity of about 55 seconds. Mutations of *per* lead to alterations in the period length of this ultradian rhythm, a phenomenon discussed fully in Chapter 4. In the present context, two points are worth noting. First, genetic mosaic studies showed that song phenotype was determined by *per* expression in the thoracic ganglion and not the brain (Konopka et al., 1996); although the neural circuitry for song production resides in the thoracic ganglion (Ewing, 1979; von Schilcher and Hall, 1979), *per* is not expressed in neurons, but in glial cells (Ewer et al., 1992). Second, this work again reveals that there is a relationship between ultradian and circadian rhythms; there are interesting parallels between the rhythmicities in courtship song and in bioluminescence (see Section D5 above).

7. Mating

The preceding sections have documented detailed circadian mechanisms by which male and female insects attract each other at specific times of a circadian cycle using mating-specific signals. The operation of these mechanisms inevitably leads to a consequential periodicity of mating behaviour. It is generally accepted that the rhythm of mating is driven by a circadian oscillator, although there have been few true circadian studies. None of these requires the interpretation that mating is not a consequence of circadian control over the events that preceded it.

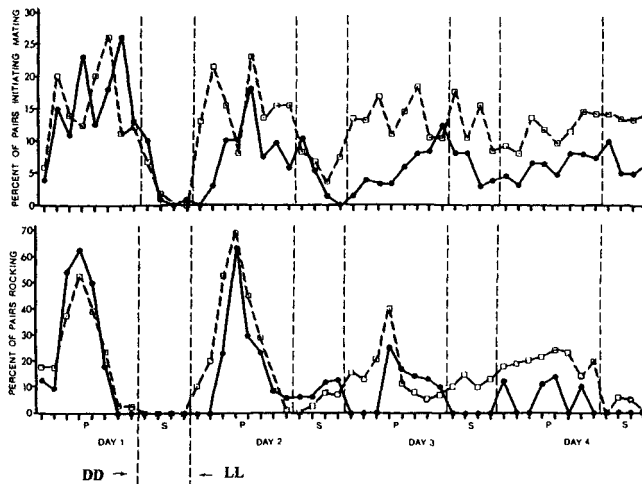


Fig. 5.31. Circadian rhythms of initiation of mating (upper panel) and rocking behaviour during copulation (lower panel) of the milkweed bug, *Oncopeltus fasciatus*. Both rhythms free-run for 2-3 cycles in DD (solid line) and LL (dashed line). (After Walker, 1979).

In the milkweed bug *Oncopeltus fasciatus* (Walker, 1979), various component behaviours of mating were shown to possess a circadian component; the initiation and termination of copulation and rocking behaviour of females during mating all persisted for

several cycles in both DD and LL (Fig. 5.31). In the beetle *Carydon serratus* (Boucher and Pierre, 1988), the fruit flies *Dacus oleae* (Loher and Zervas, 1979) and *Dacus tryoni* (Tychsen and Fletcher, 1971) and the moth *Ancylis sativa* (Han et al., 2000) rhythmicity in mating behaviours were all found to show degrees of persistence in LL or DD. However, the authors note that mating normally occurs at dusk and is suppressed by either, or both, bright light or darkness. Such observations are suggestive of direct responses of the insects to light or dark (i.e. exogenous or 'masking' effects, see Chapter 2), which are superimposed on underlying circadian control. The presence of both exogenous and endogenous elements regulating the rhythmicity of mating behaviour might be explained by a model in which the behaviours that bring the sexes together are under circadian control, whereas mating behaviour itself occurs at a preferred light intensity, most commonly the dim light of dusk. This dim light lasts for less than an hour each day; the direct effects of light would serve to constrain the time available for mating to a narrower window than could be achieved by circadian control alone. Sakai and Ishida (2001) found that the rhythm of mating in female *Drosophila melanogaster* was abolished in *per* and *tim* null mutants, emphasising the presence of underlying circadian control. The rhythm was also lost in *disco* mutants, which have severe defects in the optic lobes and lack the lateral pacemaker neurons (see Chapter 4). Thus, behaviourally arrhythmic flies are unable to mate rhythmically. This finding is consistent with the possibility that the locomotor activity clock in the optic lobe may regulate rhythmicity in mating behaviour. Sakai and Ishida (2000) suggest the rhythm in mating may be a consequence of circadian control over pheromone release and/or responsiveness to the male courtship song.

8. Oviposition

The deposition of eggs on a substrate (oviposition) is under circadian control in numerous species (see Chapter 3). Where a large number of eggs is matured following a single mating, batches of eggs are usually deposited within a narrow window of time each day for several days. This pattern of oviposition has provided favourable material with which to conduct analysis of the formal properties of the circadian clock that controls oviposition. In many species, individual insects oviposit with a circadian rhythm, enabling oviposition to be studied either as an individual animal rhythm (Chapter 2) or as a population rhythm (Chapter 3), or as both (Ampleford and Davey, 1986). A circadian oviposition rhythm has been described in moths (Bell, 1981; Riedl and Loher, 1980; Shearer et al., 1995; Yamaoka and Hirao, 1981), crickets (Sefiani, 1987), bugs (Ampleford and Davey, 1986; Constantinou, 1984) and flies and mosquitoes (Allemand, 1983; Joshi, 1996).

Mating accelerates the onset of oviposition. In moths such as *Bombyx mori* which develop all their eggs in one batch and then die, mating is followed by oviposition of all the eggs within 24 hours. If mating does not occur, the eggs can be retained in the female for many days. In some species, unfertilised eggs are eventually deposited. Therefore, mating affects the *timing* of oviposition but is not essential for its occurrence. In *Rhodnius prolixus*, the oviposition of fertilised eggs is under circadian control (Ampleford and Davey, 1986), whereas that of unfertilised eggs is arrhythmic. It seems that events consequent upon mating are necessary for activating the clock that controls the oviposition rhythm.

The physiological links between mating and oviposition are both complex and variable between species. The material transferred to the female at mating may provide nutrients to the female and thereby accelerate oogenesis; amino acids and even proteins, from spermatophores are found in the eggs of various insects (Huignard, 1983; Mullins et al., 1992). In tettigoniids and crickets, the female eats the spermatophore, the proteinaceous components of which can be

found later in the eggs (Simmons and Gwynne, 1993). Other peptides and proteins transferred to the female at mating are known to enhance fertility, promote oviposition and inhibit further mating by the female (Kubli, 1992; Stanley-Samuelson, 1994). Thus, the male contributes to the completion of egg development and to the changes in behaviour that follow mating. These observations further emphasise the close functional associations between reproductive behaviours and egg development.

The circadian activation of oviposition requires not only circadian control of the behaviour, but also synchronised circadian control of the presence and movement of eggs along the oviducts. The neuroendocrine mechanisms underlying these processes have been extensively studied in orthopterans. The TAG contains a central pattern generator (Thompson, 1986a) that innervates the ovipositor and drives the rhythmic muscular contractions associated with digging. The motor neurons to the ovipositor muscles release proctolin and the pathway is modulated by octopamine (Belanger and Orchard, 1993). The oviduct muscle possesses a myogenic rhythm of contraction, which is co-ordinated by a second pattern generator and, again modulated by octopamine (Facciponte and Lange, 1992; Kalogianni and Theophilidis, 1993; Cheung et al., 1994). Proctolin (Noronha and Lange, 1997) and peptides related to FMRF-amide (Orchard et al., 1997) also participate in the regulation of contraction of the oviducts. An appropriate point at which clock control could provide inputs to both digging behaviour and contraction of the oviducts is at the central pattern generators in abdominal ganglia or at their command neurons in the head or thorax (Thompson, 1986b; Yamaoka and Hirao, 1997). Circadian activation of the motor programmes would lead to rhythmicity in the pathways for release of neuropeptides and octopamine. None of the neurophysiological studies conducted to date has examined the circadian control of these pathways.

Dedication and Acknowledgement

Respectfully dedicated to the memory of Colin Pittendrigh, who shared with us his many thoughts on the future of insect chronobiology and our glasses of ouzo. Research in the authors' laboratory is supported by the Natural Sciences and Engineering Research Council of Canada.

ANNOTATED SUMMARY

1. This chapter addresses the functional organisation of circadian mechanisms, with emphasis on intercellular communication and the many roles of the neuroendocrine system as components of timekeeping systems and in the communication and coordination of outputs from such systems.
2. Numerous cellular oscillators throughout the insect body are coordinated by nerves and hormones. The developmental hormones prothoracicotropic hormone (PTTH), ecdysteroids and the juvenile hormones (JHs) are released throughout life, form a key component of the circadian system and may represent the central timekeeping system.
3. The neuropeptide PTTH is released rhythmically from the brain complex in *Bombyx*, *Periplaneta* and *Rhodnius* under the control of 'clock cells' in the protocerebrum. The prothoracic gland (PG) cells that synthesise ecdysteroids are also 'clock cells'. In *Rhodnius*, PG cells are directly photosensitive and synthesise ecdysteroids with a circadian rhythm, creating a rhythm in the haemolymph. PTTH acts on the PGs to control the phase of rhythmic ecdysteroidogenesis; therefore, PTTH couples brain and PG clocks into a multioscillator timing system that regulates rhythmicity in ecdysteroids. All developing

cells possess ecdysteroid receptors, which also cycle, enabling rhythmic gene transcription. The ubiquity of cellular responses to ecdysteroids suggests the multioscillator system that regulates them may represent the central timing system of insects. These hormones are present in adult insects, where this timing function may also operate.

4. The behaviour of shedding the old cuticle (ecdysis) is usually gated and the control mechanisms have been extensively studied. Ecdysteroids provide information for timing ecdysis by regulation of the synthesis and release of ecdysis-specific hormones and the responsiveness of the nervous system to these hormones.
5. The cuticle secreted by epidermal cells during moulting contains microfibrils of chitin, the orientation of which changes during a day and may result in the formation of daily growth layers. In locusts, the epidermis is said to be photosensitive and to contain a clock that regulates circadian formation of growth layers. Ecdysteroids may contribute to this rhythmicity in other species. Hatching of larvae from the egg is frequently under circadian control of a clock that becomes functional at 50-60 per cent through embryonic development, when PER protein first appears in the brain.
6. The physiology of adult insects is organised around reproduction, numerous aspects of which are under circadian control. These processes are controlled and coordinated by the same set of hormones that act during larval development, raising the prospect of a continued operation of the multioscillator timing system in adults. In adults, both PTTH cells and the 'clock cells' that regulate them persist from the larval stages. The corpora allata receive neural input from 'clock cells' in the brain; JH secretion may be rhythmic in adults. Ecdysteroids are secreted by the gonads, but rhythmicity has not yet been examined.
7. The physiology of oogenesis, ovulation and egg transport are thoroughly studied and known to involve several hormones and neural mechanisms. Although the end result of these processes is a circadian rhythm of oviposition in many species, circadian elements in the control mechanisms have not been explored.
8. In moths, the release and transport of sperm is under circadian control by a clock within the reproductive system. *per* is expressed in epidermal cells of the vas deferens, but does not free-run. The roles of nerves and hormones in regulating observed rhythmicity in the duct musculature awaits examination.
9. The release of sex pheromones is commonly under circadian control. This ensures that males and females are brought together at appropriate times of day for mating and also synchronises members of a population with each other. Pheromone release is usually accompanied by 'calling behaviour' and often by a rhythm of pheromone responsiveness in the other sex. Pheromone production in moths involves JH, pheromone biosynthesis activating peptide (PBAN) and sometimes octopamine. PBAN is released rhythmically into the haemolymph but may also be delivered locally to pheromone glands by abdominal nerves. An octopaminergic innervation may also be present. Rhythmicity in PBAN and/or octopamine is driven from within the CNS.
10. Antennae of *Drosophila* possess a *per* based circadian oscillator that is necessary for circadian changes in olfactory responsiveness. In some moths, octopamine modulates the responsiveness of antennal neurons to sex pheromone. Sensory inputs from the antennae appear to be relayed to the locomotor clock in the brain and employed to modulate flight, courtship and mating behaviours.
11. Mate location by emission of flashes of light is best known in fireflies. Flashing is regulated by octopaminergic neurons from the last two abdominal ganglia. Flashing occurs with a circadian rhythm that is synchronised with the rhythms of visual responsiveness and flight activity. The flashing pattern is repeated as an ultradian rhythm that can be modelled

in synchronously flashing fireflies as a population of interacting photosensitive oscillators which are synchronised by mutual entrainment. The oscillators for both circadian and ultradian rhythms are in the brain.

12. Mate location by sound emission is well studied in crickets and grasshoppers. Stridulation is under circadian control by oscillator(s) in the optic lobes that likely time the activation of protocerebral command neurons to the stridulation pattern generators in the thoracic ganglia. The sound emitted may be modified to a courtship song when the sexes make visual contact. In *Drosophila*, both the circadian and ultradian rhythmicities in the courtship song involve *per* expression.
13. Many more behaviours related to reproduction are under circadian control, including mating behaviour and oviposition. Many of these are dependent upon the prior, appropriately timed, production of mature eggs and sperm. Many of these rhythms are regarded as consequential rhythms that are ultimately timed by the hormones that control gamete production. These are the same hormones that regulate development, implying they and the putative central timing system of which they are part, function throughout insect life.

CHAPTER 6

THE MULTIOSCILLATOR CIRCADIAN SYSTEM

My own suspicion is that the universe is not only queerer than we suppose, but queerer than we can suppose. J.B.S. Haldane

CONTENTS

Introduction	189
<i>A. Evidence for Separate Oscillators in Cells and Tissues</i>	190
<i>B. Evidence for more than one Oscillator Governing Overt Rhythmicity</i>	191
1. Bimodality, and the 'splitting' phenomenon	191
2. Possible role of ultradian rhythms as components of circadian rhythms	195
3. Spontaneous changes of τ , and so-called 'after-effects'	196
4. Duplication of pacemaker structure as a consequence of bilateral symmetry	198
5. The hierarchical arrangement of pacemakers and slaves	200
6. Different pacemakers with different functions	204
7. 'Larval' and 'adult' clocks	205
<i>C. Environmental Periodicity and Fundamental Aspects of Physiology</i>	208
1. Survival	208
2. Rate of Development	210
Annotated Summary	212

INTRODUCTION

THE foregoing chapters show that insects display a large number of rhythms (of activity, behaviour and physiology) which have evolved with a close match to the period of the earth's rotation around its axis. Many of these rhythms have an adaptive significance in that - in their entrained steady state - they attain a particular phase relationship to the environmental cycle so that the insects perform certain functions at particular times of the day or night. One of the fundamental questions about this temporal organisation is the nature of this '*circadian system*', particularly whether there is a single (central) 'master clock' somewhere in the body driving all the observed rhythms, or whether organisms are a 'population' of clocks.

The idea of a single driving oscillation (a 'master clock'), entrained by the light-cycle on the one hand, and hierarchically coupled to a number of overt circadian activities on the other, appears to be an outmoded and almost certainly erroneous concept. Authors now agree that the circadian system of a multicellular organism (such as an insect) consists of a number -

possibly a large number - of oscillators or pacemakers, each associated with a number of driven rhythms controlling behavioural or physiological phenomena. Since unicellular algae and other single celled organisms display circadian rhythms, it has been suggested that every cell within the body of a multicellular organism may be, or may contain, its own 'clock'. However, animals such as the insects are not merely aggregations of cells: just as morphological differentiation has occurred to produce tissues and organs with a variety of functions, differentiation has also occurred to produce tissues and organs with specific time-keeping or time-measuring ability. Thus, although the cell may retain an ancestral 'clock', different tissues and organs may contain autonomous or semi-autonomous pacemakers. In this sense, the tissue or organ may be said to contain an identifiable circadian pacemaker *for that particular function*. In the case of behavioural rhythms, such as locomotor activity or pupal eclosion, it is not surprising that such pacemakers are to be found in the brain or in an associated part of the central nervous system; the sites of such clocks, their photoreceptors and the nature of their outputs will be reviewed in Chapter 8. The present chapter will describe the evidence for multiple clocks in different tissues.

The evidence for this multioscillator 'construction' of the circadian system comes from a variety of observations to be described below. It is now known that circadian pacemakers are to be found in a variety of organs other than the brain and optic lobe; these include sense organs, endocrine glands, gonads, malpighian tubules, epidermis, and possibly 'every' cell in the insect's body. Here the material is drawn together to underline the importance of the concept of a multioscillatory circadian system, and to provide a basis for later discussion of the nature of photoperiodic time measurement (Chapter 13).

A. EVIDENCE FOR SEPARATE OSCILLATORS IN CELLS AND TISSUES

The biochemical and cellular feedback loops considered to be central to the mechanism of the circadian clock are discussed in Chapter 4. For many years, however, it has been known that some single-celled organisms (such as the marine dinoflagellate *Gonyaulax polyedra*) may show an array of overt rhythms (e.g. photosynthesis, cell division, luminescent glow and stimulated flashing) originally regarded as being coupled to a single pacemaker (Sweeney, 1969). The rhythms are not pacemakers themselves: photosynthesis, for example, may be blocked by the inhibitor DCMU (dichlorophenyl dimethyl urea) but, after its removal, the rhythm resumes in phase with the rhythm before treatment (Hastings, 1970). Similarly, the antibiotic puromycin inhibits the rhythm of spontaneous glow but the rhythm re-appears in phase after the drug's removal. The conclusion was that all four rhythms were driven slaves coupled to a central pacemaker. Recent evidence, however, has shown that even single-celled organisms such as *Gonyaulax* may contain more than one pacemaker (Roenneberg and Morse, 1993); it remains clear, however, that the circadian clock is essentially a cellular phenomenon.

Evidence that individual cells of insects are similarly clocks in their own right is far less convincing and will ultimately depend on the study of single isolated cells for its demonstration. Rhythms of cuticle deposition (reviewed in Chapter 5, A), however, may present an intriguing example since the deposition of cuticle is, of course, the biochemical responsibility of the cell immediately beneath it. Recent work with the cockroach *Blaberus craniifer* (Lukat et al., 1989; Weber, 1995) has demonstrated the independence of such a rhythm from the central nervous system or endocrine milieu. In addition Plautz et al. (1997) have recently used transgenic *Drosophila melanogaster* expressing either luciferase or green

fluorescent protein to demonstrate circadian rhythms in a wide range of cells and tissues including those underlying legs and wing bristles.

Konopka and Wells (1981) demonstrated circadian control of cell division in cultured cells of *Drosophila melanogaster*. In low amplitude temperature cycles (8 hours at 24°, 16 hours at 20°C; or 12 hours at 24°, 12 hours at 20°C) cells were found to divide in a bimodal fashion during the warm phase, with an 'anticipation' of the warm onset in the shorter thermoperiod. After transfer to constant conditions (DD, 20°C) the rhythm persisted for at least two cycles thus indicating its endogeneity.

At the organ and tissue level there are several examples of persistent rhythmicity in excised structures, notably the isolated eyes of the marine mollusc *Aplysia* (Jacklet, 1969) and the isolated pineal organ of the sparrow (Binkley, 1979). Among the insects, the salivary glands of *Drosophila melanogaster* retain free-running bimodal rhythms of nuclear (Rensing, 1969) and chromosome puff size (Rensing et al., 1973) for several days *in vitro*. Rhythms in tissues as diverse as endocrine organs (Vafopoulou and Steel, 1996; Emery et al., 1997), sense organs (Meinhertzhagen and Pyza, 1996), gonads (Giebultowicz et al., 1989) and malpighian tubules (Hege et al., 1997) have also been described. These are reviewed in Chapter 5. In many cases these oscillators appear to be independent of the central nervous system, but are well-developed oscillators, self-sustained and photoreceptive (Giebultowicz, 1999). Whether they are to be regarded as circadian pacemakers or slaves is a moot point to be discussed later. In any event, the concept of a central *master* clock is dead and buried.

There seems little reason to doubt that cells and organs of insects, as well as other animals, are capable of independent circadian oscillations.

B. EVIDENCE FOR MORE THAN ONE OSCILLATOR GOVERNING OVERT RHYTHMICITY

Largely for the sake of simplicity, the properties of overt behavioural and physiological rhythms were described in Chapters 2 and 3 in terms of a 'single' oscillator entraining to the environmental cycles of light and temperature. However, many of the properties of these rhythms can only be explained in terms of more than one, and in some cases in terms of many, constituent subsystems, together making up the total multioscillator 'circadian system', and providing an all-pervading temporal organisation for the animal. Experimental evidence in favour of such a proposition includes the following: the 'splitting' of activity periods into two or more components; spontaneous changes in the circadian period, τ ; 'after-effects' of entrainment or illumination on τ ; and the evidence that the circadian pacemaker is structurally complex, either as a result of bilateral symmetry, of a hierarchical arrangement of pacemakers and 'slaves', or of different pacemakers governing different functions, sometimes at different periods of development.

1. Bimodality, and the 'splitting' phenomenon

Many insect rhythms are bimodal (see Chapter 2, B. 5), frequently with peaks of activity close to dawn and dusk. Such patterns, however, are not necessarily evidence for more than one 'component' in the clock: bimodality might merely reflect a single oscillator with two active phases, or a situation in which overt rhythmicity is suppressed, for example, by the masking effect of above-threshold daytime light intensity. Satisfactory evidence for the existence of separate 'dawn' and 'dusk' (or 'morning' and 'evening') oscillators is provided by

either (1) stably different phase relationships between the activity peaks, established during entrainment to cycles containing different durations of light, and retained during subsequent DD free-run, or (2) the 'splitting' of the activity band into two or more components which, at least for a while, free-run with different periods. In the most convincing cases the separate components may span the entire 360° of mutual phase relationship, and may even cross and recross. These cases are often referred to as 'internal desynchronisation'.

The first requirement is at least partly met in several insect species. In the mosquito *Aedes aegypti*, for example, the flight activity rhythm was found to be bimodal with peaks close to dusk and dawn. When transferred from LD 4:20 to DD both peaks persisted, with the phase relationship established during entrainment to the light-cycle (Taylor and Jones, 1969).

Internal desynchronisation was first encountered in the arctic rodent *Spermophilus undulatus* maintained for extended periods in LL. In the absence of a periodic *Zeitgeber* the rhythm of locomotor activity not only free-ran, but after a while split spontaneously into two diverging components one of which ran with a lower frequency (higher τ value) until it rejoined the main band of activity, having scanned the full 24-hour period of the cycle (Pittendrigh, 1960). Similar phenomena have since been observed in other rodents (Pittendrigh, 1974; Pittendrigh and Daan, 1976b), the tree shrew *Tupaia belangeri* (Hoffmann, 1971), the starling *Sturnus vulgaris* (Gwinner, 1974) and a lizard *Sceloporus olivaceus* (Underwood, 1977). Desynchronisation may be brought about by a variety of treatments: exposure to LL of various intensities (rodents, tree shrews), transfer from LL to DD (rodents), the use of testosterone treatments (starling), or pinealectomy (lizard). In rodent examples where activity is frequently bimodal, the 'morning' and 'evening' peaks are seen to diverge and may attain a stable phase relationship about 180° apart. In pinealectomised lizards, however, the two components may cross and re-cross, spanning the 360° of phase relationships many times over. The differences presumably concern the presence and strength of the coupling mechanism between the constituent oscillators, which in *Sceloporus* may be provided by the pineal organ.

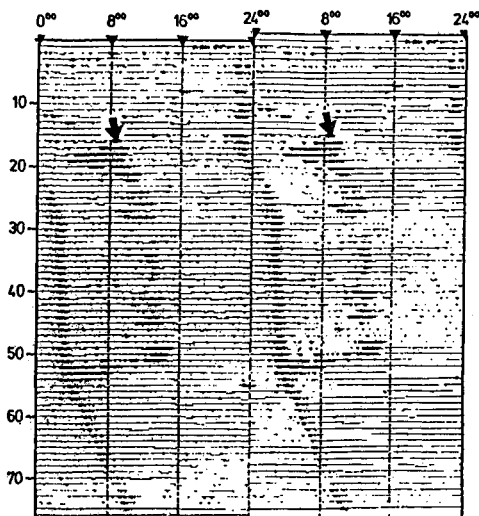


Fig. 6.1. *Leucophaea maderae*, locomotor activity rhythm. An example of 'splitting' of the activity rhythm in constant conditions (continuous red light, $170 \mu\text{W cm}^{-2}$). The free-running period (τ) changes abruptly after the split and again after subsequent merging. (From Wiedenmann, 1980).

In insects, the dissociation of locomotor activity rhythms has been recorded in cockroaches (Pittendrigh, 1974; Wiedemann, 1977a; Thomson, 1976), but the results are generally less clear than the vertebrate examples outlined above. In *Leucophaea maderae*, for example, a split in the activity band may appear spontaneously in red light (Fig. 6.1), or after disturbance by a temperature or light-pulse (Wiedemann, 1977a), and in *Nauphoeta cinerea* it has been observed in LL (Thomson, 1976). In most cases, however, the second activity peak never departs slowly from the main band: it shows its full phase angle difference (usually about 180°) immediately, and both 'components' then free-run with the same period. For these reasons the phenomenon in cockroaches may not be comparable to that described above for rodents and other vertebrates. Indeed, bimodality in the cockroach *L. maderae* has been attributed to a single circadian pacemaker (Wiedemann, 1980).

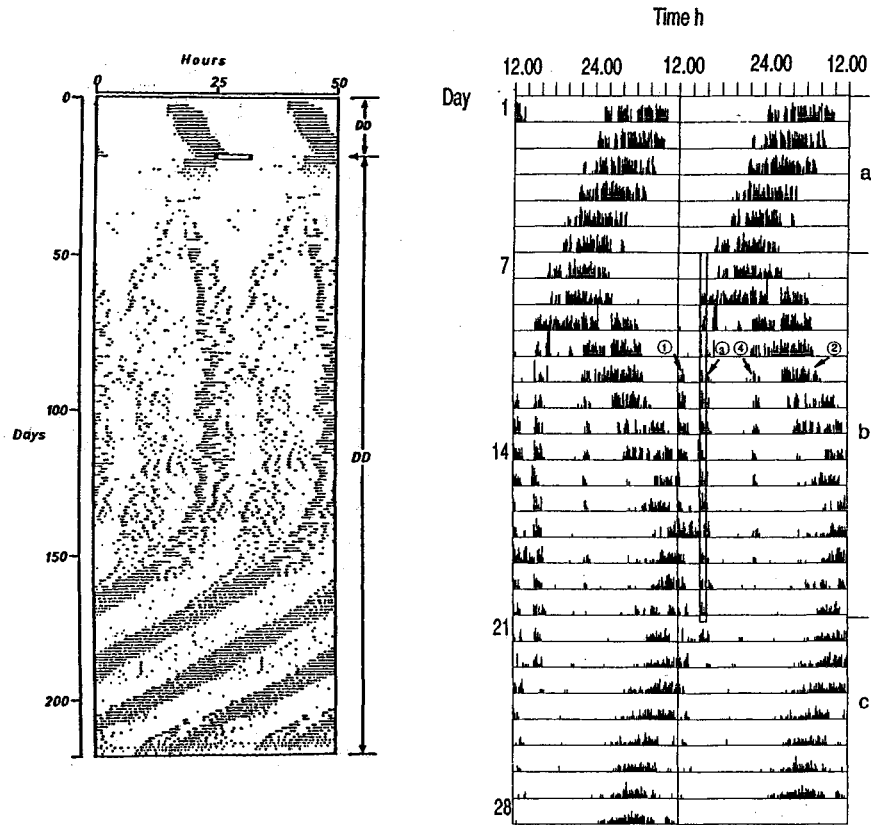


Fig. 6.2. (left). *Hemideina thoracica*. 'Splitting' of the locomotor activity rhythm in constant conditions (DD) following perturbation by a single light pulse. The activity 'band' splits into a number of components that eventually coalesce after about 150 days. (From Christensen and Lewis, 1982).

Fig. 6.3 (right). *Calliphora vicina*. Locomotor activity rhythm initially free-running in DD (20°C). Starting on day 8 the fly was exposed to a cycle of LD 1:23 with the daily 1 hour light pulse commencing in the middle of the subjective night, and continuing until day 21. The light pulses caused 'splitting' of the locomotor activity rhythm into at least four components (numbered 1 to 4) that either ran with an altered value of τ or entrained to the LD 1:23 cycle with different phase relationships (see text for details). (From Hong and Saunders, 1998).

Christensen and Lewis (1982), however, encountered genuine desynchronisation of the locomotor activity rhythm in the New Zealand weta, *Hemideina thoracica*. In this species (Fig. 6.2) splitting into a number of components occurred shortly after exposure to a light pulse, and the different components free-ran in DD, sometimes with different periods, for over 100 days before finally coalescing into a discrete band of activity. This observation can only be accounted for by assuming that the locomotor activity rhythm is controlled by several, if not a large number of circadian pacemakers.

Internal desynchronisation of locomotor activity rhythms has also been observed in adult flies. Smietanko and Engelmann (1989) showed that house flies (*Musca domestica*) maintained under low illumination LL, produced a high proportion (63 per cent) of complex rhythms which increased further when 1mM LiCl was added to the drinking water. These complex patterns were interpreted as a reflection of interactions within a group of oscillators, some with a shorter period, some with a longer period, normally coupled to give a common circadian output. Locomotor rhythms of the blow fly, *Calliphora vicina*, also showed splitting and internal desynchronisation in about 5 per cent of cases (Kenny and Saunders, 1991; Hong and Saunders, 1998). When blow flies were exposed to a light cycle of LD 1:23 with the 1 hour light pulses commencing in the middle of the subjective night, some flies showed a form of internal desynchronisation in which the daily activity 'band' broke up into several components. In the example shown (Fig. 6.3) four such components were evident: one (1) entraining to LD 1:23 with a phase lead to the light pulses, one (4) entraining with a phase lag, one major band (2) running with $\tau > 24$ hours until it appeared to entrain, and one (3) directly coinciding with the light pulses. As in *M. domestica*, these various components are probably the outputs of constituent circadian oscillations with different periods which become separately evident when mutual coupling between them is weakened. The separate components may reflect uncoupled outputs from lateral 'clock' neurons in the brain (see Chapters 4 and 8).

Multiple circadian oscillators contributing to the main activity band have also been proposed for the cricket, *Teleogryllus commodus* (Rence, 1984; Wiedenmann and Loher, 1984) and the mosquito, *Culex pipiens pallens* (Chiba et al., 1993). Recent and important observations on the locomotor activity rhythm of *Drosophila melanogaster* (Hamblen-Coyle et al., 1992; Wheeler et al., 1993; Helfrich-Förster, 2001) have indicated the 'separate' nature of the 'morning' and 'evening' peaks, with the former probably independent of the *period* gene (see also Chapters 2 and 4).

Working with *Hemideina thoracica*, Christensen and Lewis (1982) described aperiodic locomotor behaviour closely resembling that which might be caused by a light-pulse failing on the oscillation's 'singularity' (see Chapter 3, E), but in an *individual* insect rather than in a population. Figure 6.4 shows an example in which a 12-hour light pulse commencing at about Ct 18 caused 'destruction' of the normal circadian rhythm of activity. Locomotor behaviour after the pulse was clearly erratic, but whether this reflected a 'true' arrhythmicity following the stopping of the clock by a singular stimulus, or the scattering of phases within a population of oscillators (i.e. splitting) is a moot point. But since subsequent exposure to a light-cycle (LD 8:16) appeared to reunite scattered bursts of activity into a single band, and this reunification seems to take at least 3 days, splitting rather than true arrhythmicity may be the preferred conclusion (see Chapter 7).

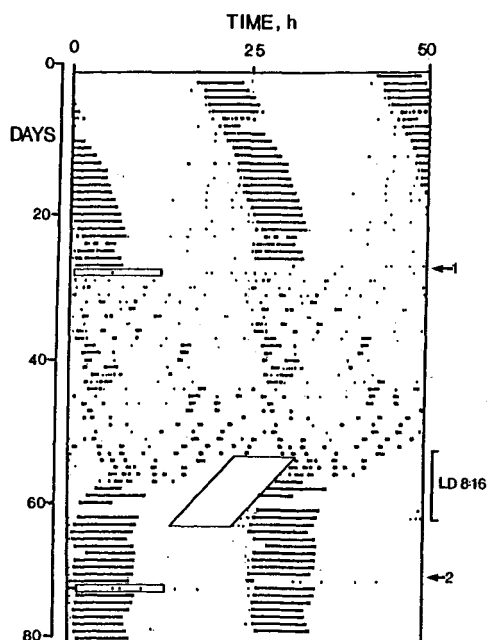


Fig. 6.4. *Hemideina thoracica*. Locomotor activity rhythm showing the appearance of arrhythmicity following a 12 hour light pulse (1). Rhythmicity is re-initiated after exposure to a light cycle (LD 8:16). A second attempt to produce arrhythmicity by a 12 hour pulse (at 2) failed to do so even though it was delivered at the same phase as (1). (From Christensen and Lewis, 1982).

2. Possible role of ultradian rhythms as components of circadian rhythms

Although the *per*^O mutant of *Drosophila melanogaster* is often considered basically arrhythmic (see Chapter 4), several studies have uncovered weak circadian and ultradian (periods shorter than circadian) rhythms in the apparently arrhythmic records. For example, using sensitive digital techniques for signal analysis (correlograms and MESA - Maximum Entropy Spectral Analysis), Dowse et al. (1987) uncovered both circadian and ultradian components in the activity records of *per*^O males, and in females entirely lacking the *per* gene (*per*⁻) because of overlapping deletions of the *per* locus. Dominant periods were from 4 to 22 hours, most flies showing multiple periodicities. It was hypothesised that the *per* gene product may act to couple multiple ultradian oscillators into a coherent circadian rhythm in the wild type fly. The circadian output would be a function of coupling strength, period (τ) decreasing as coupling tightens: *per*^O being the loosest and noisiest, *per*^L and *per*⁺ less so, and *per*^S the most tightly coupled (Dowse and Ringo, 1987).

It was also shown that rearing *D. melanogaster* wild-type flies in constant darkness for several generations produced phenocopies of circadian clock mutants (Dowse and Ringo, 1989). Only 23 per cent of such flies showed wild-type rhythms, some with clear ultradian components. A further 68 per cent were apparently phenocopies of either *per*^O (52 per cent) or *per*^L (16 per cent). The most frequent phenotype was *per*^O-like without rhythms, whereas a

further 16 per cent were *per*^O-like with significant ultradians. It was suggested that exposure to light during development somehow helped to couple the population of ultradian rhythms into a coherent, circadian clock. Occasional examples of ultradian rhythms in locomotor activity patterns have been described in other insects, for example the blow fly, *Calliphora vicina* (Kenny and Saunders, 1991).

Residual rhythmicity in apparently arrhythmic *per*^O flies was also uncovered by Helfrich and Engelmann (1987) and McCabe and Birley (1998). In the first of these studies, wild type and *per*^O flies were exposed to light-dark cycles with periods ranging from $T = 19$ to $T = 27$ hours. In both strains it was found that the 'band' of locomotor activity phase-lagged the light when T was short, but phase-led the light when it was long. These characteristic phase angles indicated 'true' entrainment of an internal rhythm rather than mere 'forcing' of behaviour by the light cycle (see Chapter 2 and 3). More than 60 per cent of the *per*^O flies entrained to cycles of $T = 19$ and $T = 27$ hours. McCabe and Birley (1998) later examined both locomotor activity and oviposition rhythms in wild type, *per*^S, *per*^L and *per*^O flies, in LD 12:12, LL and DD, looking for endogenous periodicity (and phase angle to the light, signal-to-noise ratio and genetic penetrance). In *per*^O, significant rhythms were found for locomotor activity, for example, in both LL ($\tau = 19.3$ hours) and DD ($\tau = 18.6$ hours). Both of these studies, therefore, indicated some, albeit weak, rhythmicity remaining in *per*^O flies.

3. Spontaneous changes of τ , and so-called 'after-effects'

Spontaneous changes in the period of locomotor rhythms in rodents have been known for some time, sometimes in association with ageing (Pittendrigh and Daan, 1974). Abrupt period changes in constant conditions have also been described for insects, including the cockroach *Nauphoeta cinerea* (Thomson, 1976) and the New Zealand weta, *Hemideina thoracica* (Christensen and Lewis, 1982) (see Fig. 2.3). It is generally agreed that such changes suggest that the circadian system is a complex of many oscillators (Daan and Berde, 1978).

A similar conclusion may be drawn from a study of a class of phenomena called 'after-effects' (Pittendrigh, 1960), in which the free-running period τ is altered following a variety of treatments. These include entrainment to cycles where T is not equal to τ ; entrainment to cycles ($T = 24$ hours) containing different photophases; entrainment to skeleton photoperiods; phase shifting by single light-pulses; and following an exposure to constant light (Pittendrigh and Daan, 1976b). For example, in the mouse *Mus musculus* and the hamster *Mesocricetus auratus*, τ was short following entrainment to T less than τ , but long following entrainment to T greater than τ ; in the first case it gradually lengthened to its 'normal' value (τ_{DD}), and in the latter it gradually shortened. In the white-footed deer mouse *Peromyscus leucopus*, τ_{DD} was longer than expected following exposure to LL and then gradually shortened, yet it was shorter following entrainment to long photoperiods.

Comparable phenomena have now been described for insect activity rhythms. In the cricket *Teleogryllus commodus*, for example, τ following exposure to continuous red light was longer than expected, but gradually shortened over the next 3 weeks in DD (Sokolove, 1975). In addition, nine out of thirteen examples showed an altered value of τ following exposure to a single resetting light pulse. Once again, however, the clearest examples concern the weta *Hemideina thoracica* (Christensen and Lewis, 1982). Figure 6.5 for example, shows the effects of entrainment to light-cycles with different T -values. Following entrainment to a long T cycle (LD 8:23, $T = 31$ hours, or LD 8:21, $T = 29$ hours) the free-running period (τ) was at first long but then shortened to a value close to its original. After each period of entrainment, τ was

longer than before but then gradually shortened to approach its 'normal' value in DD. In *H. thoracica* these after-effects may last for 40 days. After-effects of prior entrainment to $T \neq 24$ hours have also been described for *Leucophaea maderae* (Page and Block, 1980).

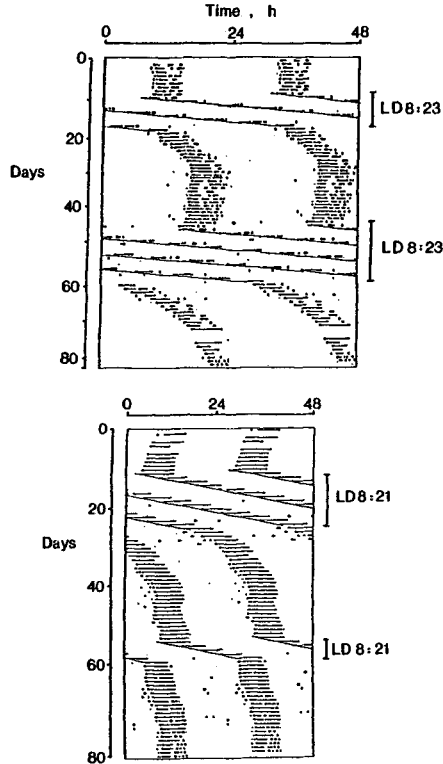


Fig. 6.5. *Hemideina thoracica*, locomotor activity rhythm. 'After effects' of entrainment to a light cycle ($T > 24$). After each period of entrainment (to LD 8:21 or 8:23) the free-running period (τ) is initially greater than that before entrainment, but then subsequently shortens. (From Christensen and Lewis, 1982).

After-effects of entrainment to 24-hour light-dark cycles have more recently been reported for the blow fly, *Calliphora vicina* (Kenny and Saunders, 1991). Newly emerged flies were entrained to light cycles (LD 4:20 to LD 20:4) for 3 to 7 days before transfer to DD. These flies showed an initial short free-running period ($\tau < 24$ hours) before an abrupt lengthening to $\tau > 24$ hours after 6 to 10 days. In some long-lived flies, these after-effects lasted for 30 days or more before a final return to a short period characteristic of a fly in darkness (Fig. 6.6). Such after-effects were never observed in flies maintained throughout adult life in DD. As with similar phenomena in rodents, these long-lasting changes in period cannot be accounted for in terms of a *single* oscillation: they are compelling evidence for a complex (or multioscillator) circadian system.

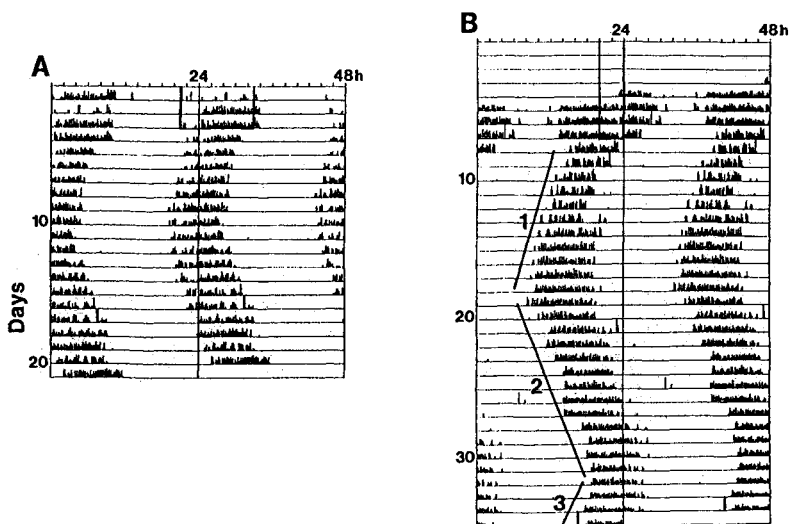


Fig. 6.6. *Calliphora vicina*. Free-running locomotor activity in DD at 20°C, following initial entrainment to (A) LD 12:12 and (B) LD 4:20. Following LD entrainment the free-running activity passed through a stage of short period activity (stage 1) followed by a region of long period activity (stage 2), and finally shortened again (stage 3). These period changes are not seen in flies in continuous darkness and are considered to be long-lasting 'after effects' of photoperiod. (From Kenny and Saunders, 1991).

4. Duplication of pacemaker structure as a consequence of bilateral symmetry

In the cockroach *Leucophaea maderae*, covering the compound eyes with opaque material (Roberts, 1965a) or cutting the optic nerves (Nishiitsutsuji-Uwo and Pittendrigh, 1968a), led to a free-running rhythmicity, even in a light-cycle, similar to that in DD. Surgical isolation of the optic lobes, on the other hand, resulted in behavioural arrhythmicity (Nishiitsutsuji-Uwo and Pittendrigh, 1968b)(see Chapter 8). The compound eyes are therefore considered to be the principal photoreceptors for entrainment, and the optic lobes the site of *two* clocks within the central nervous system. In a series of elegant experiments involving the unilateral excision or lesioning of the optic lobes, the nature of the coupling between the two putative circadian pacemakers has been examined.

Page et al. (1977) excised or surgically isolated one optic lobe of *L. maderae* leaving the other intact. It was found that either left or right lobe was sufficient to maintain a free-running rhythm (in DD), but the mean circadian periods of such insects were longer (left lobe, $\tau = 23.95$ hours; right lobe, $\tau = 23.96$ hours) than those of intact animals ($\tau = 23.73$ hours) (Fig. 6.7). This result suggested that, although either lobe was 'redundant' in one sense, the two optic lobes were normally coupled and mutually accelerated each other. They also found that insects with one optic nerve cut could entrain to a light-cycle (LD 4:21, T 25 hours). But the activity record of such an insect (Fig. 6.7) showed no evidence that one optic lobe pacemaker was entrained whilst the other was free-running: light received by either photoreceptor could entrain ipsilateral and contralateral pacemakers, again suggesting mutual coupling, perhaps by some sort of reciprocal excitation.

The apparent lack of mutual coupling between the eyes of the beetle *Blaps gigas* (Koehler and Fleissner, 1978) could not provide a more dramatic contrast, bearing in mind, of course, that the eyes are more peripheral structures, and optic lobe pacemakers may well control locomotor rhythms in these insects. At intervals of 30 minutes the eyes of *B. gigas* were exposed to 30-msec flashes of light and the electroretinogram (ERG) recorded.

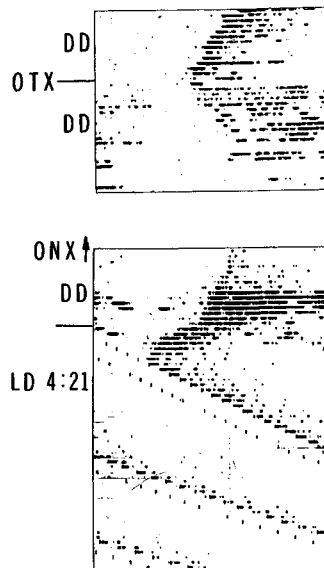


Fig. 6.7. *Leucophaea maderae*. Upper panel: increase in τ after unilateral section of the optic tract (OTX) showing that τ for a single optic lobe pacemaker is greater than that for the two bilaterally arranged pacemakers combined. Lower panel: entrainment of the locomotor rhythm to LD 4:21 ($T = 25$ hours) in an insect with unilateral optic nerve section (ONX) showing *no* evidence that one optic lobe pacemaker was entrained whilst that isolated from its eye was free-running. (From Page et al. 1977).

These ERGs, caused by a circadian pupillary response were found to oscillate in a circadian fashion, with the eyes being 10 to 100 times more sensitive during the subjective night than the subjective day. In the cockroach *L. maderae* the two optic lobes remained in synchrony in DD free-run, even when one lobe was separated from its ipsilateral photoreceptor (Page et al., 1977). In *Blaps*, however, the two eyes frequently drifted out of synchrony after a few days in DD; in some cases the two oscillators spanned the entire 360° of mutual phase relationship (Fig. 6.8 upper left) thus meeting the criterion for fully independent circadian pacemakers. The independence of the eyes was further underlined in experiments in which only one eye was illuminated (with fibre optics). When one eye was exposed to LL and the other to DD, the period of the former became longer (in accordance with Aschoff's rule; Chapter 2) (Fig. 6.8). And when one eye was exposed to LD 12:12 and the other to DD, the former entrained, whilst the latter did not (Fig. 6.8). There seems little doubt that the compound eyes of *B. gigas* constitute bilaterally organised circadian oscillators which are largely, if not entirely, free from mutual entrainment. Unlike *L. maderae*, therefore, each eye acts as a photoreceptor for its own clock. Synchrony between the two eyes must be controlled

by the environmental Zeitgeber. Further examples of independent, bilaterally organised, circadian pacemakers in the insect eye are discussed in Chapter 5.

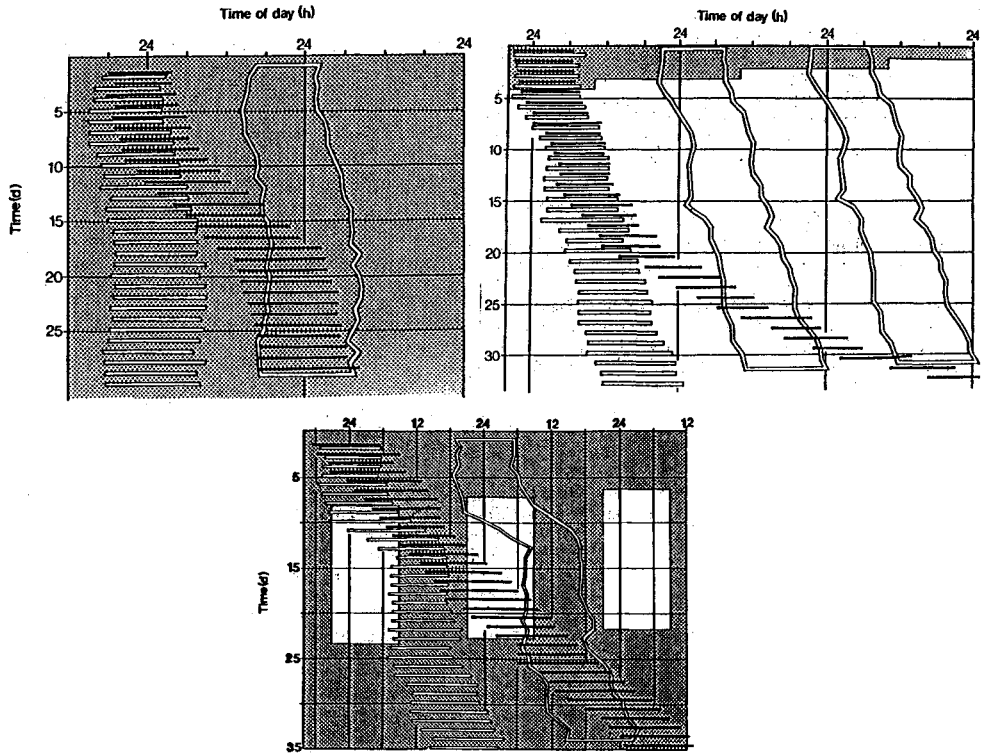


Fig. 6.8 (upper left). *Blaps gigas*. Electrophoretogram (ERG) measured, for both eyes separately, by applying 30 msec flashes of light at intervals of 30 minutes. Horizontal white and black bars show the times of heightened sensitivity (~subjective night) for the two eyes. After several days in DD the two eyes drift out of phase with each other, the two oscillators spanning the entire 360° of mutual phase relationships.

(upper right). One eye in DD, the other illuminated by fibre optics (LL). The ERGs of both eyes free-run independently, with that of the illuminated eye (black bars) adopting a much longer period ($\tau \sim 28$ hours) and spanning two complete cycles of the DD eye.

(lower). One eye in DD (black bars), the other exposed to an LD cycle by fibre optics (white bars). The latter entrains to the light cycle, the former does not, indicating almost complete independence of the two circadian pacemakers. (From Koehler and Fleissner, 1978).

5. The hierarchical arrangement of pacemaker and slaves

The first evidence that the circadian clock might involve a complex *hierarchical* or 'vertical' arrangement of coupled oscillators was obtained from resetting experiments with the pupal eclosion rhythm of *Drosophila pseudoobscura* (Pittendrigh and Bruce, 1957, 1959; Pittendrigh et al., 1958; Chapter 3). These experiments showed that single pulses of light produced changes in phase but, in doing so, brought about transient cycles which, especially in

the case of phase advances, took several days to subside (see Fig. 3.17). Although certain single oscillator models may explain such transients, other properties of the system cannot. The most persuasive explanation is that the physiological mechanism underlying eclosion consists of a two-tier arrangement of a circadian *pacemaker* (or A-oscillator), and a driven *slave* (or B-oscillator) which is coupled to the pacemaker on the one hand and to the overtly rhythmic event (eclosion) on the other (Pittendrigh, 1967b) (see footnote*). The pacemaker is 'immediately' reset by light (and temperature) pulses and is 'coupled' by means of the entrainment phenomenon to these environmental *Zeitgeber*, whereas the 'slave' rhythm is not light sensitive but may be sensitive to temperature changes. The pacemaker is also self-sustained and therefore free-runs in DD, whereas the 'slave' may be rapidly damped without reinforcement from the driver. In this model the non-steady state or transient cycles which follow perturbation (see Fig. 3.17) arise as a consequence of the coupling mechanism between pacemaker and slave.

Experimental evidence for such a model will receive further consideration here. In *D. pseudoobscura* two pieces of evidence are the most compelling. Two-pulse resetting experiments (Chandrashekar, 1967; Pittendrigh, 1974), already described in Chapter 3, demonstrated that light pulses reset the central pacemaker virtually instantaneously and that the observed transients must be, therefore, a feature of a more peripheral system. Pittendrigh and Minis (1971) later showed that the phase relationship between the eclosion peak (the slave) and its pacemaker (as defined by the PRC) was dependent on the period of the *Zeitgeber* (T hours) as defined by recurrent 15 minute pulses of light. Figure 6.9, for example, shows steady state phase relationships of pupal eclosion (ψ_{RL}) and its pacemaker (ψ_{OL}) to T cycles between 19 and 30 hours. As expected (see Chapter 3), eclosion *phase-led* the *Zeitgeber* when T was greater than τ but *phase-lagged* it when T was less than τ . More importantly, however, the eclosion peak (phase reference point for the overt rhythm, or slave) phase led its pacemaker when $T > \tau$, but phase lagged it when $T < \tau$. Such an observation can only be explained by postulating two hierarchically arranged oscillators, a light sensitive oscillation or pacemaker coupled to a driven element, or slave. In a later paper (Pittendrigh, 1981a) demonstrated such a flexible coupling between pacemaker and slave in cultures exposed to different temperatures and photophases (Fig. 6.10).

Selection for 'early' and 'late' eclosion strains of *D. pseudoobscura* (Pittendrigh and Minis, 1971; see also Chapter 3) also provided evidence for a two-tier circadian system governing adult emergence. Fifty generations of selection (performed under LD 12:12) gave rise to an 'early' strain emerging several hours before a 'late' strain under all photophases (Fig. 6.11). When phase response curves for the divergent strains were determined, they were found to be *identical*, both to each other and to their parental stock. Since the PRC characterises the underlying, light entrainable oscillation, the observed differences between 'early' and 'late' can only be attributed to a driven element or slave and to the flexible coupling between slave and pacemaker. Furthermore, the phases of eclosion between the selected strains 'early' and 'late' were also flexible. 'Early' phase-led 'late' at high temperature but, below about 13°C, a *phase reversal* occurred so that 'late' occurred *before* 'early'. This further underlined the fact that the

*Over the years, Pittendrigh changed the designations of pacemaker and slave. For the sake of the reader, these are listed here (see also the Glossary). The 'driving' *pacemaker* was variously denoted as *oscillator* (O), *pacemaker* (P) or *A-oscillator* (A). The 'driven' *slave* was referred to as *rhythm* (R), *slave* (S), *B-oscillator* (B) or simply *eclosion* (E). The phase relationship between pacemaker and light cycle was then, for example, either ψ_{OL} or ψ_{PL} , the phase relationship between slave (rhythm) and light ψ_{RL} and that between slave and pacemaker was ψ_{RO} , ψ_{SP} , ψ_{EP} or ψ_{AB} . Happy reading!

gating signal that times the flies' eclosion behaviour is not associated with any fixed phase point in the pacemaker's cycle.

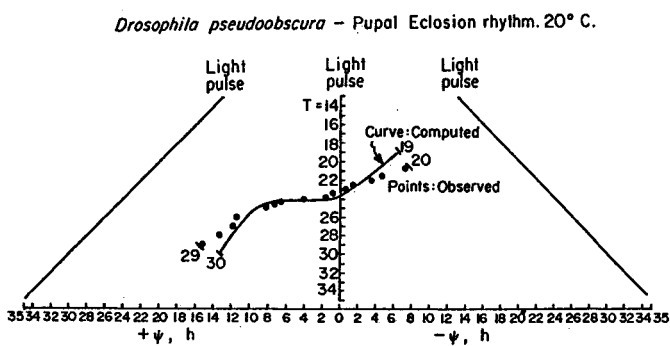


Fig. 6.9. *Drosophila pseudoobscura*. The steady state phases of the pupal eclosion rhythm (ψ_{RL}) and its oscillation (ψ_{OL}) to the period (T hours) of the light cycle defined by a single 15 minute light pulse per cycle. The solid curve marks ψ_{OL} ; it is a predicted curve (see Pittendrigh, 1965) which has been confirmed experimentally (see Pittendrigh, 1967). The points mark the phase of the rhythm. When $T > \tau$ the rhythm (slave) phase leads the oscillation (pacemaker); when $T < \tau$ the rhythm (slave) phase lags the oscillation (pacemaker). (From Pittendrigh and Minis, 1971).

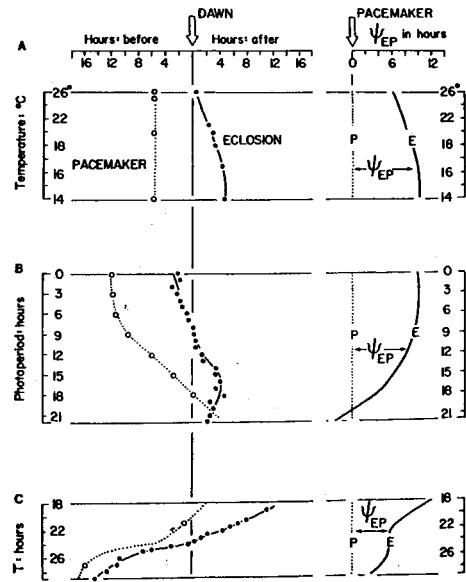


Fig. 6.10. *Drosophila pseudoobscura*. The phase relationship (ψ_{EP}) between eclosion peak (E) and its pacemaker (P) is shown to be labile. A – it is temperature dependent, becoming more negative at lower temperatures; B – it changes with photophase ($T = 24$ hours), becoming more positive as photophase lengthens; C – it becomes more negative as the pacemaker's period shortens because of entrainment by an external LD cycle (period T). (From Pittendrigh, 1981b).

Computer simulations of such phenomena (Pittendrigh, 1981b) have suggested that the observed lability of the phase relationship between eclosion and pacemaker (ψ_{EP}) is dependent on three parameters: ρ (or the ratio of τ_B/τ_A), the damping coefficient of the slave (ϵ), and the coupling strength (C) between slave and pacemaker. In the simulation shown in Fig. 6.12A, in which ϵ was held constant, two coupling strengths were compared. Holding τ_A (pacemaker period) at 24 hours, an increase in τ_B (and hence an increase in ρ) led to a characteristic phase reversal, very similar to that shown in Fig. 6.12B for populations of 'early' and 'late' as temperature decreased. Thus, at lower temperature, ρ would increase because τ_B was less temperature-compensated than τ_A , this leading to delayed eclosion. The model was further developed to consist of a pacemaker coupled to a number of slave oscillations with different values of ρ , ϵ and C ; phase relationships among these slaves were found to vary within themselves as these parameters changed. In 1981 Pittendrigh (1981a) suggested that "...any feedback loop in the organism is a potential slave oscillator, and if the circadian pacemaker can make input to the loop, the slaves will become part of the temporal program that the pacemaker drives".

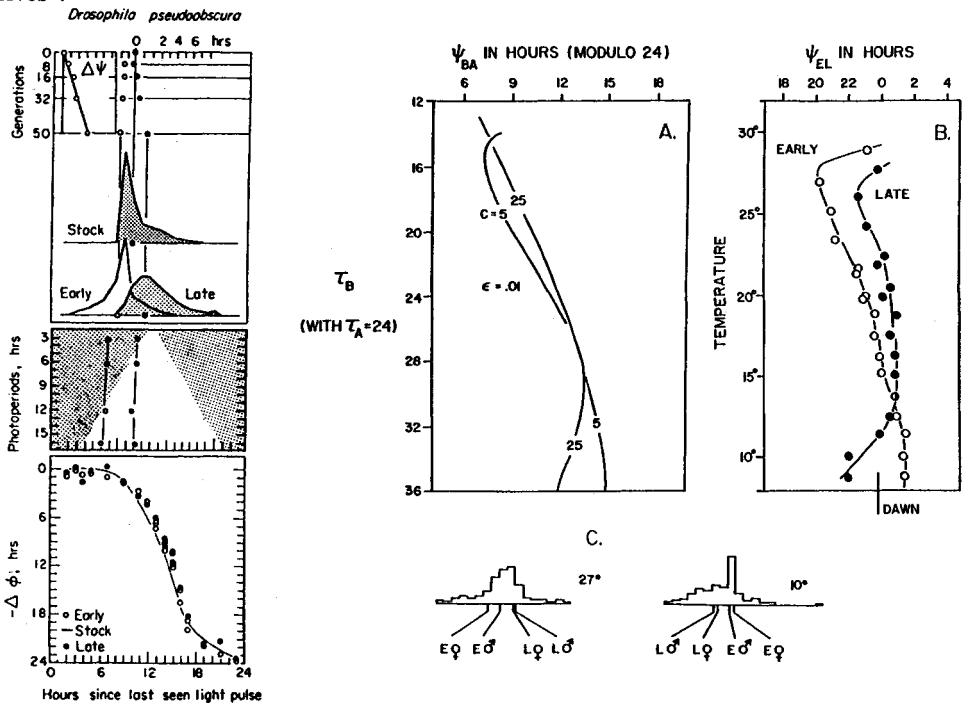


Fig. 6.11 (left). *Drosophila pseudoobscura*, eclosion rhythm. The effect of selection for 'early' and 'late' eclosion strains. Top – the change in ψ_{RL} for the two strains through 50 generations of selection. Middle panel – the ψ_{RL} for the two strains in 5 photoperiods; Lower panel – the phase response curves for 'early' (open circles) and 'late' (solid circles) compared with that from the stock culture (solid line), showing that selection had affected ψ_{RL} but not ψ_{OL} (From Pittendrigh and Minis, 1971).

Fig. 6.12 (right). *Drosophila pseudoobscura*. Phase reversals within the eclosion peak as τ_B lengthens at lower temperatures. A – ψ_{BA} for two slave oscillators with the same damping coefficient ($\epsilon = 1.01$) but differing in C . As τ_B is lengthened and the ratio of τ_B/τ_A passes from <1 to >1 their phase relation is inverted. B – the effect of

temperature on the phase relation of *Early* and *Late* to the light pulse (15 minutes) in a 24 hour LD cycle. At higher temperatures *Early* is earlier than *Late*, but as temperature is lowered their phase relation is inverted. This is predicted if τ_B is lengthened at lower temperatures. C – the eclosion peaks (at 27 and 10°C) of the synthetic population created by mixing *Early* and *Late*. At 27°C the phase sequence is: *Early* females, *Early* males, *Late* females, *Late* males. At 10°C the entire sequence is inverted. (From Pittendrigh, 1981c).

Van Gelder et al. (1995) described 20 genes isolated from the heads of *D. melanogaster* which produced mRNAs with a greater than 2 fold daily change in their abundance. These genes were called *Dregs* (*Drosophila* rhythmically expressed genes) (also considered in Chapter 4). Three of them were expressed in the morning, each with a unique dependence on the light cycle and function of the *period* (*per*) gene; 17 were most abundant in the evening. Some of the genes (*Dreg1*, 3, 6-10, 15) continued to oscillate in DD with a slightly reduced amplitude. These genes and the proteins they express were considered to be on the ‘output pathway’ from the *per* ‘clock’ loop, whose function was to regulate the flies’ circadian behaviour. Could they also be components of their own feedback loops, damped and temperature dependent, which are slaves to the *per-tim* pacemaker?

Outside *Drosophila*, data suggesting the existence of a pacemaker-slave structure to the circadian system may be evident in several species of Orthoptera, following surgical removal of the optic lobes, the known site of the pacemaker for locomotion and singing rhythms (see Chapter 8). For example, in the crickets *Teleogryllus commodus* (Rence and Loher, 1975) and *Gryllus bimaculatus* (Tomioka, 1985), and in the cockroaches *Blaberus fuscus* (Lukat and Weber, 1978) and *Leucophaea maderae* (Page, 1983, 1985), bilateral lobectomy generally led to behavioural arrhythmicity (in DD), but rhythmic singing or locomotion persisted in some individuals, or after the imposition of a daily temperature cycle. These results could be evidence for an oscillatory structure - with some of the properties of a slave (damping, temperature entrainment) - outside the optic lobes, perhaps in the protocerebrum.

What of the ‘physiological’ rhythms (sometimes of unknown function) that have been described in epidermal cells (Lukat et al., 1989), testes (Giebultowicz et al., 1989), malpighian tubules (Giebultowicz and Hege, 1997; Hege et al., 1997) and prothoracic glands (Mizoguchi and Ishizaki, 1982; Emery et al., 1997; Vafopoulou and Steel, 1996, 1997) (see Chapter 5)? Are these pacemakers or slaves? Most of them appear to be independent of the brain or central nervous system, and most of them are entrained directly by the light, and are probably persistent and temperature compensated. Therefore, they are probably best regarded as pacemakers. However, the phase of the ecdysteroid rhythm emanating from *Rhodnius* prothoracic glands is influenced (‘entrained’?) by secretion of prothoracotropic hormone (PTTH) (Vafopoulou and Steel, 1997), suggesting a brain-prothoracic gland axis resembling a pacemaker-slave hierarchy. Perhaps a too rigid interpretation using the *Drosophila* eclosion rhythm model is inappropriate in these cases.

6. Different pacemakers with different functions

In earlier sections (Chapter 5) we have seen examples of circadian pacemakers governing ‘physiological’ rhythms in tissues and organs such as epidermal cells, gonads, malpighian tubules and endocrine glands which are apparently largely independent of influences from the brain and CNS. There are also cases where several overt behavioural and physiological rhythms are observed in the same species, often with quite different circadian parameters, indicating that the pacemakers driving them are separate and distinct. In the most

compelling examples, the apparently different circadian clocks are observed to run concurrently, at the same stage of development.

In the pink boll-worm moth, *Pectinophora gossypiella*, Pittendrigh and Minis (1971) described three rhythmic functions: egg hatching, pupal eclosion and oviposition. In steady-state entrainment to the daily light-dark cycle the three rhythms achieved different but characteristic phase relationships to the light; egg hatching and eclosion occurred during the early part of the day and oviposition at night. When transferred to DD, however, eclosion and oviposition revealed free-running periods (τ_{DD}) of about 22.5 hours, whereas τ_{DD} for the egg-hatch rhythm was much closer to 24 hours. Furthermore, red light failed to entrain either eclosion or oviposition, but perceptibly shortened τ for eclosion. More recently, Giebultowicz and Zdarek (1996) have shown that the rhythm of sperm release from the testis and the rhythm of mating flight in two species of gypsy moth (*Lymantria dispar* and *L. monarcha*) presented quite different properties. These observations may be taken as evidence for more than one pacemaker in the circadian system.

Among the Diptera, multiple circadian clocks have been described in the flesh fly *Sarcophaga argyrostoma* (Saunders, 1986) and the blow fly *Lucilia cuprina* (Warman, 1995). In *S. argyrostoma*, the departure of the fully fed larvae from their food (larval 'exodus' behaviour) was shown to occur at night, whereas pupal eclosion occurred at dawn, and adult locomotor activity was diurnal. When transferred to continuous darkness, all three rhythms persisted, but the free-running period (τ_{DD}) for larval exodus was close to 20 hours, whereas those for eclosion and locomotor activity were very close to 24 hours. In *L. cuprina*, larval exodus was nocturnal with $\tau_{DD} \sim 23.4$ hours, eclosion occurred near dawn with τ_{DD} close to 24 hours, and adult locomotor rhythmicity occurred during the day, but with τ_{DD} about 22.5 hours. These data all point to a complex of different pacemakers in the insects' circadian system.

The different types of circadian pacemaker in insects were classified into two groups (Truman, 1971d). In Type I, which includes 'physiological' or 'developmental' rhythms such as pupal eclosion, hatching, chitin lamellogenesis, hormone release, etc., light seems to bypass the organised photoreceptors (eyes, etc.) and impinge directly on the relevant tissue. Sensitivity to the phase-shifting effects of light appears to be high, giving rise to Type 0 phase-response curves (Chapter 3, C. 1) and frequently to arrhythmia in LL of moderate intensity. In Type II clocks, which control 'behavioural' rhythms such as locomotor activity, on the other hand, light is frequently perceived by the organised photoreceptors (e.g. the compound eyes in cockroaches), and the clock's responses to light include low 'amplitude' (Type I) phase-response curves and persistence in LL. Truman included photoperiodism (Chapter 9) in his Type I and sun compass orientation and the bees' time sense (Chapter 15) in Type II, mainly because the former seemed more akin to developmental rhythms and the latter to behavioural activity. Although many of these seemingly clear distinctions have become blurred in recent years, Truman's classification still has merit.

7. 'Larval' and 'adult' clocks

In many insects, particularly those with pronounced changes at metamorphosis, there seems to be an ontogenetic distinction between 'larval' and 'adult' clocks. The former are frequently 'developmental' clocks in Truman's sense and may be classified as Type I, and adult clocks are frequently 'behavioural' or Type II. In some species the larval and adult clocks have quite distinct properties; the adult clocks may also begin to operate after larval clocks have

ceased, or both may be present and even functional during the course of larval, pupal and adult differentiation.

In several species of mosquitoes larval clocks 'gate' pupation (and hence cause periodic eclosion of the adults; Chapter 3) whereas adult clocks govern flight activity. Working with *Aedes taeniorhynchus*, Nayar (1967 a, b) and Nayar and Sauerman (1971) showed that the pupation rhythm was entrained during the larval stages but free-ran in DD with a period (τ) of about 21.5 hours. The clock governing adult flight activity, however, appeared to originate in the adult stage, about 36 hours after eclosion, and to free-run in DD with τ of about 23.5 hours. In *Culex pipiens pallens*, on the other hand, the adult flight clock seemed to start just before emergence (Chiba, 1966), and in *Anopheles gambiae*, Jones and Reiter (1975) demonstrated that the flight rhythm may be entrained by the light-cycle as early as the second larval instar. In this species, therefore, the adult clock shows a considerable 'overlap' with the larval clock controlling pupation.

Although Konopka and Benzer (1971) showed that a single gene (*period*) on the X-chromosome was an important element in controlling pupal eclosion and adult locomotor activity rhythms of *Drosophila melanogaster* (see Chapter 4), more recent data of Engelmann and Mack (1978) indicated that these clocks in *D. pseudoobscura* not only 'overlap' in the pupal instar, but present quite distinct properties. The eclosion rhythm, for example, free-ran in DD with a period close to 24 hours, showed the characteristic high 'amplitude' Type 0 PRC (Winfree, 1970) to short (55 seconds) light-pulses, and a phase angle (ψ) between light-off and the eclosion peaks of about $15 + \text{modulo } \tau$ hours. The activity rhythm, on the other hand, free-ran with τ_{DD} of about 22.5 hours, showed a ψ -value of about zero, and a low-'amplitude' Type 1 PRC in response to pulses as long as 3 hours (Fig. 6.13). The shapes of the PRCs were also quite different, with that for adult activity lacking the so-called 'dead-zone' for light pulses falling during the middle of the subjective day.

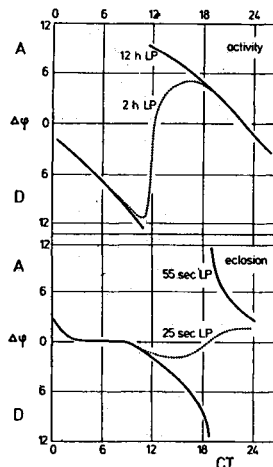


Fig. 6.13. *Drosophila pseudoobscura*. Comparisons between phase response curves (PRCs) to light pulses for (A) the adult activity rhythm and (B) the pupal eclosion rhythm. PRCs for activity are 'strong' for 12 hour pulses (white light, 7000 lux) but 'weak' for 2 hour pulses. Those for eclosion are 'strong' for 55 second pulses, but 'weak' for 25 seconds. The shapes of the curves and the positions of the major phase changes are also different in the two systems. (From Engelmann and Mack, 1978).

Step-wise transfers of single flies from LL to DD, at different stages of their development, indicated that both clocks were functional in the intra-pupal stage (Engelmann and Mack, 1978). For example, in transfers of flies to darkness a few hours *after* eclosion, or 5 days *before* eclosion, onsets of locomotor activity in both cases could be extrapolated back to the point of transfer (Fig. 6.14), showing that adult activity as well as pupal eclosion could be initiated in the pharate adult. The two separate clocks, therefore, were functional at the same time.

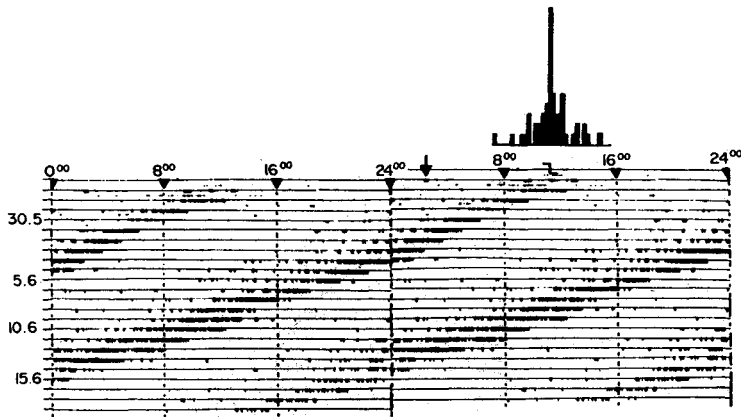


Fig. 6.14. *Drosophila pseudoobscura*. Free-running activity rhythm of a female fly at 22°C, continuous red light. $\tau = 22.1$ hours. Vertical arrow – time of eclosion. Step down shows the time of transfer from LL (white light) to weak red light. Activity onsets extrapolate back to the LL/RR transition rather than to the time of eclosion. Different clocks are thought to control eclosion and adult activities. (From Engelmann and Mack, 1978).

Outside the Diptera, evidence for separate larval and adult clocks is available for at least two species. In the pink boll-worm moth *Pectinophora gossypiella*, Pittendrigh and Minis (1971) found that the two ‘adult’ rhythms (pupal eclosion and oviposition) showed an endogenous period of about 22.5 hours, whereas a developmental rhythm (egg hatch) showed τ_{DD} of about 24 hours. In the pupal eclosion and adult flight activity rhythms of giant silkmoths we have some information on the anatomical location of the photoreceptors, the clocks, and the nature of their output (see Chapter 8), as well as their more formal properties. In *Antheraea pernyi* the eclosion rhythm free-ran with τ_{DD} of about 22 hours, both the photoreceptor and the clock were in the cerebral lobes, and the output was humoral (Truman and Riddiford, 1970). The flight activity rhythm in *A. pernyi*, *Hyalophora cecropia* and *Samia cynthia* also free-ran in DD, photoreceptors and clocks were also brain-centred, but the output to the thoracic motor centres was neuronal (Truman, 1974). Thus there are certainly differences between the developmental (eclosion) and the behavioural (flight activity) clocks in these species, but how far these differences reflect ‘larval’ versus ‘adult’ clocks, or Type I versus Type II, remains to be seen.

C. ENVIRONMENTAL PERIODICITY AND FUNDAMENTAL ASPECTS OF PHYSIOLOGY

Organisms maintained in conditions of constant light and temperature, or in environmental cycles with a periodicity far from that which matches the earth's rotation, sometimes show impaired growth and survival, or deleterious changes affecting general 'fitness'. Many plants, for example, become abnormal and necrotic when maintained in LL and constant temperature (Arthur and Harwill, 1937; Hillman, 1956) or show sub-optimal growth rates in light-cycles longer or shorter than 24 hours (Highkin and Hanson, 1954; Went, 1959). This section examines similar phenomena in insects.

1. Survival

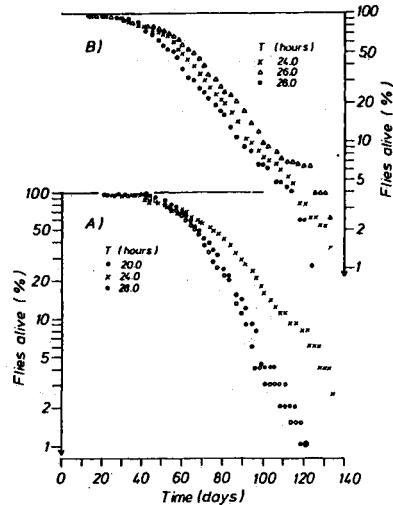
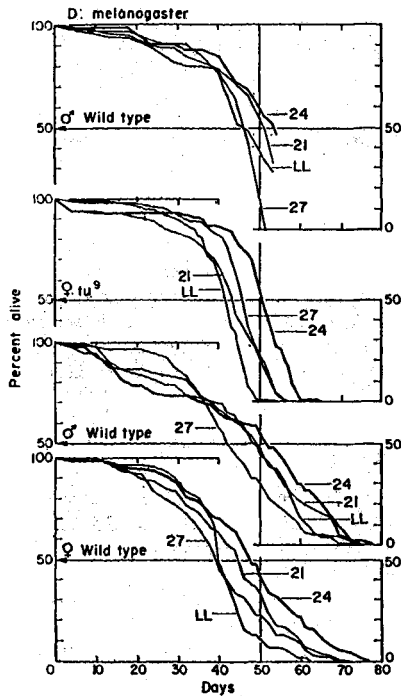


Fig. 6.15 (left). *Drosophila melanogaster*. Survival of populations of flies in light/dark cycles whose period (T) is 24, 21 or 27 hours, and in LL. In all four experiments the flies on a 24 hour day lived significantly longer than the flies in the other environments. (From Pittendrigh and Minis, 1972).

Fig. 6.16 (right). *Phormia regina*. Survivorship curves for adult flies kept in light cycles with a different Zeitgeber period (T = 24, 26 or 28 hours), each containing 50 per cent of light. A and B show results of two such experiments; in A survival is greatest at T = 24 hours. (From Saint Paul and Aschoff, 1978).

Pittendrigh and Minis (1972) kept adults of *Drosophila melanogaster* in different light-cycles (LD 12:12, T = 24 hours; LD 10.5:10.5, T = 21 hours; LD 13.5:13.5, T = 27 hours), and in LL, all at a constant temperature. In both males and females of a wild type, and females of a

tumorous strain (*tu*^B), survival was significantly greater when the flies were 'driven' at $T = 24$ hours than in the other regimes (Fig. 6.15). The amount of light received was not the factor involved because it constituted 50 per cent of the cycle in each case. The results were attributed to the fact that organisms, having evolved an innate periodicity (τ) in their metabolic functions close to the natural cycle of the earth's rotation, perform 'more efficiently' when driven by light-cycles close to τ . The deleterious effects of light-cycles far from 'circadian resonance' may be the result of internal disorder, or desynchronisation, among the component parts of the multioscillator circadian system.

Klarsfeld and Rouyer (1998) extended this work using flies of different genotype (see also Chapter 4). Males of *D. melanogaster* wild type (*per*⁺), the ultra-short *per*^T ($\tau_{DD} \sim 16$ hours) and the long period *per*^L ($\tau_{DD} \sim 28$ hours) were maintained under light-dark cycles of either LD 8:8 ($T = 16$ hours) or LD 12:12 ($T = 24$ hours) and their life spans used an index of physiological adaptation. They found that survival of the two mutant strains was reduced by up to 15 per cent, even when T closely matched τ . The LD cycle made no significant difference on its own, but an interaction between genotype and light cycle had some impact on survival. Organisms such as *D. melanogaster* appear to incur a physiological penalty from living in a light cycle that differs too widely from their endogenous periodicity.

A second example concerns longevity of the blow fly *Phormia terraenovae* in light cycles from $T = 20$ to $T = 28$ hours, and in LL (Saint Paul and Aschoff, 1978). Survival was determined as the number of days elapsing until only 10 per cent of the flies were still alive, and then expressing this figure as a percentage of survival in $T = 24$ hours (Fig. 6.16). Maximum survival was found between $T = 24$ and $T = 27$ hours, less (90 per cent) at $T = 23$ and $T = 28$ hours, still less (85 per cent) at $T = 22$ hours, and least (70 per cent) at $T = 20$ hours. In constant light, survival was between 52 and 75 per cent of that in LD 12:12 ($T = 24$ hours), depending on light intensity. That optimal survival was found in regimes where T was greater than 24 hours probably reflects the fact that τ is greater than 24 hours in *P. terraenovae*. Like Pittendrigh and Minis (1972), Saint Paul and Aschoff attributed these results to 'resonant' interactions between the period of the driver (T) and the flies' intrinsic circadian period (τ). Several reasons were considered for the reduced longevity when T was far from τ . These involved loss of entrainment to the *Zeitgeber*, internal desynchronisation, or changes in the phase angle to the light-cycle. They rejected the hypothesis that longevity concerns the 'counting' of entrained circadian cycles, since this would have meant an increased survival time in long cycles.

The effects on survival of repeated phase shifts have also been investigated. In *Phormia terraenovae*, flies maintained in LD 12:12 but subjected to weekly 6-hour shifts in the light-cycle, either advance or delay, showed a 20 per cent reduction in their life span (Aschoff et al., 1971). Harker (1958a) also reported that sub-oesophageal ganglia removed from cockroaches (*Periplaneta americana*) and implanted daily for 4 days into the haemocoels of recipients 12 hours out of phase led to the appearance of transplantable tumours in the midgut. Nishiitsutsuji-Uwo and Pittendrigh (1967) were later unable to reproduce these results, however, and their validity remains open to question.

Butler et al. (1977) and Engelmann and Mack (1978) examined the possibility of deleterious effects on survival of 'stopping the clock'. Populations of *Drosophila pseudoobscura* were rendered arrhythmic in their pupal eclosion by perturbation at the 'singularity' (see Chapter 3, E). 'Abolishing' the circadian rhythm in this manner did not appear to shorten adult life or impair the act of eclosion. Doubts remain about these experiments, however, because the 'adult' clock governing locomotor activity appears to differ from the 'larval' clock controlling eclosion (see above) and, even if it were the same, it is not

clear that the singularity is a stable one, with the pacemaker remaining throughout adult life at zero amplitude.

2. Rate of development

Saunders (1972) raised larvae of the flesh fly *Sarcophaga argyrostoma* in a wide range of light-cycles of different length ($T = 21$ to $T = 72$ hours) with 12, 14 or 16 hours of light in each. The results showed that the rate of larval development (from the deposition of the first instar larvae to puparium formation) was a function of both T and photophase. With a 12-hour photophase larval development was most protracted at $T = 24$ hours (LD 12:12) and at $T = 48$ hours (LD 12:36) and most rapid at $T = 36$ (LD 12:24) and at about $T = 60$ (LD 12:48) (Fig. 6.17). The difference in mean developmental time between larvae maintained at $T = 36$ and $T = 48$ hours was about 3 days (or 20 per cent of the total). In the case of a 14-hour photophase the length of larval development at $T = 42$ was about 4 days shorter than at $T = 48$ hours. These differences are clearly independent of the amount of light received since larvae reared at $T = 42$ (LD 14:28) completed development in about 14 days whereas those at $T = 32$ (LD 14:18), although receiving a greater total illumination, showed a mean developmental time of about 18 days. The cyclical nature of the results was interpreted as a temporal interaction between the light pulses and innate physiological oscillations within the insects.

Figure 6.17 also shows an additional effect of photophase on developmental rates (see also Chapter 9, C). Thus, at $T = 24$ hours, a short photoperiod of LD 12:12 caused a more protracted larval development than a long day length (LD 14:10 or LD 16:8). In Fig. 6.18 these results are shown in the form of an 'extended circadian topography' of the type predicted by Pittendrigh (1972), in which the contours connect points of equal larval duration. The surface of this topography clearly demonstrates the three points of protracted development and, at $T = 24$ hours, the acceleration in long days.

Tschernyshev and Afonina (1975) have also shown a reduced duration of the life cycle in LL, DD, or in LD 9.5:9.5 ($T = 19$ hours) for *Drosophila viridis* - but not for the beetle *Trogoderma glabrum*. These results were attributed to a direct relationship between the endogenous circadian system and tolerance to unusual day-night cycles.

All of the phenomena reviewed in section C suggest that organisms perform 'more efficiently' - or at least 'differently' - when driven by light-cycles whose period T is close to τ or modulo τ , and least efficiently when driven at modulo $\tau + \frac{1}{2}\tau$. It may be supposed that internal order or synchrony within a population of circadian oscillators is high when T is close to τ or multiples of τ (i.e. when the environmental cycle and the multi-oscillator circadian system are in 'resonance'), but internal disorder or asynchrony occurs when T is far from τ . Clearly these observations have a profound bearing on the interpretation of the nature of the circadian system in multicellular organisms; they are also relevant to several models for the photoperiodic clock, to be considered later.

Some circadian rhythms, such as that of cuticular lamination (Chapter 5), become arrhythmic in LL, but rhythmicity may be restored, or arrhythmicity prevented, by a 24-hour cycle of temperature. Clearly, continuous light disorganises the circadian system in some way, but a temperature cycle can maintain it. A similar effect of thermoperiod has been observed in the maintenance of 'normal' form and function in plants and animals. Hillman (1956), for example, showed that the deleterious effects of LL on tomatoes could be avoided if a 24-hour temperature cycle was imposed on the plants.

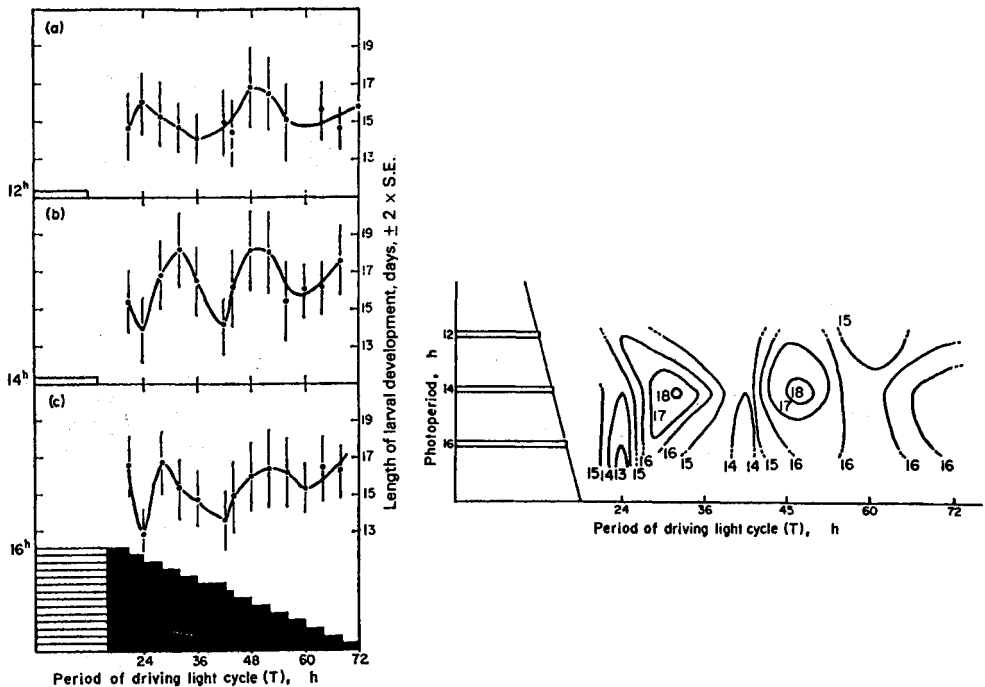


Fig. 6.17 (left). *Sarcophaga argyrostoma*. The length of larval development as a function of the period (T) of the light cycle. (a) with a 12 hour photophase; (b) with a 14 hour photophase; (c) with a 16 hour photophase. Each point represents the mean number of days to puparium formation of 200 to 900 larvae. Vertical lines indicate $\pm 2 \times \text{S.E.}$ of the mean. The white and black bars at the bottom illustrate the experimental design. (From Saunders, 1972).

Fig. 6.18 (right). *Sarcophaga argyrostoma*. Data from Fig. 6.17 redrawn as a 'circadian topography'. The contours connect points of equal larval developmental times (in days). Note the three points of protracted development, and (at T = 24 hours) the accelerated development with a 14- and 16-hour photophase. (From Saunders, 1972).

Few carefully controlled experiments of this kind have been performed with insects, although several observations support the conclusion that a temperature cycle, as opposed to a non-fluctuating environment, is beneficial. Parker (1930) found that the eggs and nymphs of *Melanoplus mexicanus* and the eggs of *Camnula pellucida* developed more rapidly in a daily thermoperiod than in the equivalent constant temperature. For example, eggs of *M. mexicanus* incubated for 16 hours a day at 22° and 8 hours a day at 32°C hatched in 6 days rather than the 8.4 days calculated for the mean temperature (25.3°C). On the other hand, those incubated for 16 hours at 12° and 8 hours at 32°C hatched in 9 days rather than the expected 15 (mean temperature 18.7°C). Messenger (1964) similarly found that a rhythmically fluctuating environment (temperature, humidity and illumination) produced the highest rates of survival, growth and reproduction in the aphid *Therioaphis maculata*. Hollingsworth (1969) maintained females of *Drosophila melanogaster* in a daily temperature cycle (25–30°C) as a test of Pearl's (1928) "rate of living" theory of ageing that maintains that if poikilotherms are kept for half

their lives at one temperature and half at another, their expected life-span would be the harmonic mean of the expectations of life at the two constant temperatures. The results showed that females lived significantly longer than predicted from this hypothesis when maintained in fluctuating temperatures, and those kept for 3 hours a day at 30°, and 21 hours a day at 25°C lived *as long* as those kept at the lower temperature throughout. Nayar (1972), working with the adults of the mosquito *Aedes taeniorhynchus*, also showed that females kept in a fluctuating temperature (12 hours at 22°, 12 hours at 27°C; mean = 24.5°C) lived slightly longer (42.4 days) than those kept at 22°C throughout (41.2 days), and significantly longer than the age-span predicted for a constant temperature of 24.5°C (~36 days). These experiments were apparently performed in a light-cycle (LD 12:12) but may be interpreted as evidence that a daily temperature cycle is beneficial in the life of insects.

ANNOTATED SUMMARY

1. The temporal organisation, or circadian system, of insects is considered to be of a multioscillator 'construction'. Rhythmicity may be observed at all levels of organisation including organs, tissues and cells. Each cell of the body may constitute its own clock, while the pacemakers controlling overt physiological and behavioural events (Chapters 2 and 3) probably consist of integrated groups of such cells within particular organs (the CNS for example) which have specific time-keeping functions.
2. Light entrainable and temperature compensated circadian pacemakers may also be found at the tissue or organ level, for example in epidermal cells, testes, malpighian tubules and endocrine glands (see Chapter 5). In many cases these 'clocks' appear to be 'independent' of the brain-centred pacemakers regulating overt behavioural rhythms.
3. Evidence for this multioscillator circadian system comes from a variety of formal observations. These include: the 'splitting' of activity rhythms; spontaneous changes in τ ; 'after-effects' of entrainment, phase-shifting, etc.; the duplication of pacemakers on the left- and right-hand sides of the body; hierarchical arrangements of pacemakers and 'slaves'; and different pacemakers with different clock properties controlling either different functions or events at different stages of development.
4. Insects appear to be best adapted to periodic environments in which T (the environmental cycle of light and temperature) is close to that of the solar day. When maintained in cycles with an unnatural period, or when subjected to frequent phase shifts, or to constant light, darkness or temperature, insects may show reduced longevity or impaired physiological function.

CHAPTER 7

QUANTITATIVE MODELS FOR INSECT CLOCKS

By R.D. Lewis, School of Biological Sciences,
University of Auckland, New Zealand

We have decided to call the entire field of control and communication theory, whether in the machine or in the animal, by the name of Cybernetics, which we form from the Greek [for] steersman. Norbert Wiener

CONTENTS

A. <i>Aims of Chronobiology</i>	214
1. Basic models for insect clocks	214
(a) The oscillator model	214
(b) Synthesis - loss model	215
(c) Master clocks and sub-clocks	216
2. Quantitative modelling	216
(a) Requirements of models	216
(b) Presentation of output	217
(c) Evaluation of the success of models	217
B. <i>The Clock as a Single Oscillator</i>	217
1. Mathematical models	218
(a) The Princeton model: Pittendrigh's empirical model	218
(b) Limit cycles and singularities: Winfree	218
(c) Van der Pol oscillators: Kronauer and Wever	219
(d) Non linear differential equations: Pavlidis	220
2. Control systems models for insect circadian clocks	221
(a) The threshold concept	224
(b) Tests of the model	224
(c) General properties of the model	224
(d) Light responses	225
(e) Temperature responses	226
C. <i>The Clock as Populations of Oscillators</i>	228
1. Free-run period lability	229
(a) Rhythm splitting	229
(b) Spontaneous changes in period	230
(c) After-effects	230
(d) Rhythm shattering	230

2. Modelling population behaviour	232
(a) Redundancy of oscillators?	235
3. Insect clocks as coupled X - Y oscillators	235
(a) X - Y characteristics of free-running rhythms	236
4. X - Y models for insect clocks	238
(a) Description of the models	238
(b) Simulations of X - Y behaviour	238
Discussion	240
D. <i>General Discussion</i>	241
Annotated Summary	242

A. AIMS OF CHRONOBIOLOGY

ONE of the central aims of chronobiology is to understand the fundamental nature of biological timing systems; their physical location, cellular and molecular organisation, and dynamic structure. In this chapter we are focussing on the understanding of the mechanistic nature of insect circadian clocks, how they work, and the quantitative models which have been proposed to describe them. Clearly this is only one facet of understanding and a full appreciation of circadian timing in insects will ultimately only be brought about by the integration of a diverse array of approaches, including control systems modelling.

INTRODUCTION

1. *Basic models for insect clocks*

(a) *The Oscillator Model*

Cursory analysis of overt free-running locomotor rhythms (e.g. Fig. 2.1) or the eclosion rhythms of insects (e.g. Fig. 3.2) tells us very little of the basic nature of the circadian timing systems underlying these rhythms. However we know that organisms exhibit self-sustained rhythms in timeless conditions, and these may be phase-controlled by external signals such as light and temperature pulses. These properties suggest that biological clocks are biochemical oscillators, and that there may be analogies between the biological oscillators underlying circadian rhythms and non-living physical oscillators. A major advance in the study of chronobiology was the hypothesis that biological clocks are living oscillators and may have the properties of their physical counterparts (Pittendrigh and Bruce, 1957). The implication is that living and non-living systems may be described by the same models, and hence could be analysed in the same terms. The application of oscillator theory to the understanding of insect circadian mechanisms was undoubtedly a watershed development that led to the unravelling of the intimacies of the covert nature of circadian systems.

The living oscillator model was the foundation of an array of descriptive and quantitative approaches to the study of the nature of insect circadian clocks and was the platform on which all research up to the current models of molecular control was carried. The basic oscillator model is very simple. The circadian system may be represented by a single endogenous oscillator that progresses through repeating phases of build up and loss on a time-scale of about a day. Physiological and behavioural rhythms are triggered as the oscillator passes through critical phases during the passage of time. Synchronisation to outside forces is

achieved as the oscillator is accelerated or slowed by perturbations falling at particular times of the circadian cycle.

(b) *Synthesis - Loss model*

The biological oscillator hypothesis envisages the regular build up and loss of an activity controlling protein on a circa 24 hour timescale, perhaps triggering activity when the concentration exceeds a threshold (Wever, 1965). Similarly eclosion may be gated when the oscillator passes through a critical concentration. The temperature sensitive build up or synthesis phase has a temperature coefficient of about 2.0, and is followed by the temperature insensitive or passive loss or degradation phase (temperature coefficient of about 1.0). As the synthesis phase is temperature sensitive, cold temperature pulses (CTPs) delay the accumulation of protein and so the threshold model can be tested. The phasing of the endogenous oscillator with the overt activity rhythm can then be deduced by observing the different effects of short CTPs administered at various times in the circadian cycle. If CTPs produce phase delays when administered at the start of the active phase we can infer that activity is promoted by the oscillating protein (Gander, 1976).

The oscillator is sensitive to external light perturbations; light may destroy or enhance the oscillating protein. In nocturnal insects it seems that the effect of light is to destroy the hypothetical oscillating protein. Light pulses delivered in the build up or synthesis phase delay the rhythm, whereas light pulses falling in the loss phase advance the rhythm (Lewis et al., 1997; Warman and Lewis, 2001). In contrast, in mammals, it appears that light leads to an increase of the concentration of the oscillating protein (Field et al., 2000).

The free-running periods of the rhythms controlled by the endogenous oscillator are rarely exactly 24 hours (See Chapters 2 and 3), and so an integral part of the functioning of the oscillator model is the entrainment of the oscillator to the 24 hour temporal patterns of the rhythmic environment. It is interesting that the circadian rhythms of insects (and other animals) which select self-imposed constant conditions during at least part of their life cycle, such as the pupal stages of insects, have free-running periods of almost exactly 24 hours. The distinctly 'circa' nature of others may well be an adaptation which allows for more precise entrainment over the seasonal cycles of shortening and lengthening days. The ecologically relevant free-running period may be quite different from the steady-state period displayed in actograms, as rhythms may go through spontaneous changes in period and characteristics during the first few days in constant conditions. The long-term period of the rhythm has no ecological significance; it is the behaviour immediately following the transference into constant conditions which is ecologically relevant.

Although it is generally assumed that the overt rhythm reflects the state of the endogenous oscillator, it has been argued that the chain of events from the hypothetical oscillator to the overt rhythm is so tortuous that nothing can be learned of the deep mechanism from the examination of the overt rhythm (Bünning, 1967). Nevertheless, the application of oscillator theory to the understanding of the mechanistic nature of circadian clocks has been of inestimable value in learning how insect circadian clocks work. Recent molecular findings which could only have been dreamed of by Bünning have reduced the links in the chain to the point that we can directly visualise and measure the covert events involved in circadian mechanisms (Hardin, 1994) (Chapter 4).

(c) Master clocks and sub-clocks

Whilst in some contexts insect clocks can be conveniently considered to be single oscillating units, the actual structure of the circadian timing system is undoubtedly considerably more complex (see Chapter 6). For example, all cells of the insect body are probably circadian oscillators, and may be grouped to form rhythmic centres. Hardin (1994) has demonstrated that body oscillators of *Drosophila* behave differently from head oscillators, and that there is a lack of *per* RNA cycling in the ovaries. Cuticle cells of cockroaches continue to display circadian rhythms of cuticle growth *in vitro* (Neville, 1983).

So it could be argued that every cell of the insect body is rhythmic and of equal standing in the structure of the insect timing system. However in contrast, ablation and transplant experiments in a range of insects (see Chapter 8), and more recent molecular genetic evidence (Renn et al., 1999), support the hypothesis that there are specialised areas of the nervous system which can be considered as pacemakers (or 'master' clocks), synchronising all the other sub-systems within the remainder of the body to the appropriate phase of the 24 h cycle by unilateral coupling. Consequently insect circadian clocks must now be seen as complex systems of interacting circadian units in which all (almost all) cells of the body are rhythmic, structured as a hierarchical system of pacemakers and slaves (or master clocks and sub-clocks).

2. Quantitative modelling

(a) Requirements of Models

Quantitative modelling should not be seen as an optional extra in the understanding of biological clocks, but should be acknowledged as an integral part of the process of unravelling their nature. Clock systems are dynamic and provide ideal opportunities to develop and test simulation models. Modelling begins with the first tentative steps of testing the internal clock hypothesis in constant conditions, followed by their entrainment by laboratory LD cycles. This should lead to the ultimate description of the nature of clocks incorporating the mechanism of control expressed as quantitative models.

The value of models should be judged not only by their success in simulating what is known about circadian rhythms. They should also be judged by the biochemical feasibility of the assumptions which made the successful simulations possible (Pavlidis and Kauzmann, 1969). Whether models should reflect the deep molecular structure of circadian clocks has been debated in the literature. Pavlidis recognises that purely mathematical models tell us nothing of the concrete nature of clock mechanisms, and sets out to present an alternative approach with the publication of a biochemical model for circadian clocks (Pavlidis and Kauzmann, 1969). Whilst this model cannot be considered to be highly successful as it came before the flood of biochemical information brought about by the molecular revolution (Chapter 4), the message it conveys was important and appropriate. It now seems inevitable that quantitative models of insect circadian clocks must be in tune with the ever-increasing molecular detail that befuddles the brain of non-molecular participants in this fascinating field.

We are aiming to create models of circadian clock mechanisms that lead to answers to the question of how biological clocks work. Quantitative models of insect clocks firstly attempt to account for known behaviour by simulating what has already been described. Successful models then create behaviour in their severely restricted worlds that mimics the essential features of the living system. A second requirement of models is to predict behaviour under new conditions that can be tested by further experimentation (Pavlidis, 1971a, 1978b).

(b) *Presentation of Output*

The simulated behaviour should be presented in such a way that it allows comparison of the simulated behaviour with real data. Rhythms of individual insects are generally presented as actograms of locomotor activity, or waveforms of physiological variables. For evaluation of model success, it seems most appropriate to present the simulated behaviour in the same form as the biological data. Since the purpose of models is in the first place to mimic the behaviour of the modelled system (at this time, the circadian clock of insects), it is logical that the output of the model is in the same format as the animal data. Most locomotor rhythms are displayed as double-plotted actograms and the model output, for the sake of easy comparison of the real and the modelled behaviour, should therefore be the same.

In the waveform presentation of the output of either a real or simulated circadian rhythm, the rhythmic variable on the Y-axis is plotted against time on the X-axis. Locomotor actograms are derived from the waveform by slicing the data into 24-hour lengths and presenting them one below the other; data are therefore plotted against time. Phase plane plots are an alternative form of visualisation of rhythmic activity, both real and simulated. In this presentation, time is not taken into account; rather two other variables are plotted against each other. One may be the concentration of the oscillating protein and plotted on the X-axis, the other the rate of change of the concentration, plotted on the Y-axis. If the oscillator is sustained then, during steady-state rhythmicity, it will move around on the path unless deflected by an external perturbation, and thus exhibit limit cycle behaviour. When the perturbation ceases the oscillator resumes the same path as before.

(c) *Evaluation of the Success of Models*

Insect rhythms are inevitably created by dynamic processes. It seems appropriate therefore to try to understand the nature of these processes by using simulation models to mimic the behaviour of insect rhythms as closely as possible. A model can be deemed to be successful if it can account for a range of behaviours of the living system, and by prediction lead to new experiments and successful confirmation. Insect clock models therefore are deterministic simulations which do not invoke stochastic (Lehmann, 1977) or chaotic (Lloyd and Lloyd, 1994) elements to account for the lability characteristic of most locomotor rhythms. The physiological significance of chaotic oscillations remains questionable with regard to circadian rhythmicity, because such behaviour is much less frequent than periodic oscillators in parameter space (Leloup et al., 1999). A model should not increase in complexity beyond what is necessary; that is the number of adjustable parameters should not increase beyond what is required to explain something, and no more assumptions should be made than are minimally needed (Ruoff et al., 1999 summarising Occam's razor).

To judge the value of models we compare the simulated output with the real data. When simulating clock behaviour there is no one single number or value or outcome which can be evaluated. Rather we compare the overall patterns of real and simulated behaviour and judge the success of the model on the basis of the congruence of the two.

B. THE CLOCK AS A SINGLE OSCILLATOR

Although the multioscillator nature of insect circadian systems is well recognised (see Chapter 6), a useful beginning to their understanding can be achieved by modelling clocks as single circadian units. The behaviour and interactions of the sub-systems can be summarised

simply as the circadian clock, and the basic nature of clock control may be understood in a broad brush fashion. The population models of circadian systems will follow later to take into account the otherwise unexplained lability of circadian rhythms.

A review of the history of Chronobiology indicates that there have been two more or less distinct approaches to quantitative modelling of circadian systems. The purely mathematical approach of non-linear differential equations (Pavlidis, 1968; Wever, 1965), and van der Pol oscillators (Kronauer et al., 1982; Jewett and Kronauer, 1998) has been developed extensively for human circadian systems. These models do not in any way represent or allude to the physical nature of the timing systems. In particular, the models do not relate to the molecular components that have more recently been identified in an impressive range of organisms.

The other approach initiated by Goodwin (1965) is to examine molecular regulatory mechanisms capable of producing sustained oscillations of the limit cycle type. Thanks to the remarkable experimental advances made in recent years on the molecular bases of circadian rhythmicity in a variety of organisms such as *Drosophila melanogaster* (Chapter 4) and the bread mould *Neurospora crassa*, the study of limit cycle models based on molecular regulatory mechanisms can be developed to a point where the state variables and the biochemical parameters of circadian clocks are largely identified in molecular terms (Goldbeter, 1995, 1996; Leloup et al. 1999; Warman and Lewis, 2001).

1. *Mathematical models*

Mathematical models for circadian rhythms were first of an abstract nature and were borrowed from the physical literature, as exemplified by the use of the van der Pol oscillator as an analogue for circadian oscillations (Wever, 1972). This approach is still used to study the effect of light on the human circadian system (Jewett and Kronauer, 1998). Subsequently, Jewett et al. (1999) replaced the van der Pol oscillator by a higher order limit cycle.

(a) *The Princeton Model: Pittendrigh's Empirical Model*

The Princeton model for the rhythm of pupal eclosion in *Drosophila* spp. (Pittendrigh and Bruce, 1957) utilises the PRC for 15 min light pulses to calculate the phase relationships under entrainment, and the use of skeleton light cycles to mimic the effect of the full photophase (see Chapter 3). This model is based on the assumption that phase changes effected by 15 minute pulses of light are completed in a short time and hence the new circadian time of subsequent pulses can be calculated. There is no reference to the concrete nature of the clock or to the genesis of the oscillation.

(b) *Limit Cycles and Singularities: Winfree*

The view that circadian rhythms may be represented by phase plane diagrams and exhibit limit cycle oscillators spans four decades (Kalmus and Wigglesworth, 1960; Pavlidis, 1968; Winfree, 1971; Leloup et al., 1999). Limit cycle topological presentation may also be interesting as it predicts the passage of the oscillator around a limit cycle, and predicts the forced movement to singularities and/or phaseless states (see Chapter 3). To achieve this the oscillator must be perturbed by a pulse of critical intensity and exact circadian time of application. This is when the PRC changes from a 'weak' Type 1 to a 'strong' Type 0 curve. The perturbation holds the oscillator at the phase where the build up or synthesis rate is equalled by the loss or degradation rate. In theory the oscillator can remain in this stationary

state 'for ever', but can be pushed from it by the smallest of perturbations. The circadian pacemaker is a limit cycle system, and the mapping described by the PRC for 15 minute high intensity light pulses takes place 'instantaneously' (Winfree, 1971).

The theory of singularities was applied by Winfree (1970) using the eclosion rhythm of *Drosophila* spp. as the oscillatory system. The eclosion rhythm of these insects, as in all others (Chapter 3), is a population rhythm where each individual subject contributes once in its lifetime to the pattern of eclosion. Each individual clock as it passes through the appropriate circadian time, opens up a gate to permit the emergence of the adult when it has reached the required stage of development. The eclosion rhythm comprises the combined action of a large number of uncoupled clocks. The behaviour of the rhythm that has been pushed from its singularity in theory depends on the strength of the pulse. A 'strong' pulse should send each oscillator to the empty Ct 18 position (see Chapter 3) and hold it there until the end of the pulse. The population rhythm will then proceed in synchrony to give rhythmic eclosion in constant conditions as each individual gate is opened. A very 'weak' pulse should push each oscillator from the singularity in a random direction, and hence lead to arrhythmic eclosion.

These two predictions of the fate of the rhythm following a strong or weak pulse administered to a motionless population are supported by the data gained for the *Drosophila* rhythm. Data from the plant *Kalanchoë blossfeldiana* (Engelmann and Johnsson, 1978), the mosquito *Culex* (Peterson, 1981) and the marine dinoflagellate *Gonyaulax polyedra* (Taylor et al., 1982) also support the concept of singularities in biological rhythms.

However, the same data also support a population model of circadian clocks. Here arrhythmicities are explained by the desynchronisation of populations of weakly coupled oscillators, each of which remains at full amplitude following the critical pulse and spontaneously resynchronises through mutual coupling with the remainder of the population. Data for the weta, *Hemideina thoracica*, may be used to support both models. As a single oscillator the responses to single critical pulses appear to support a singularity; the population data, however, explain the 'shattering' of locomotor rhythmicity in individual insects through internal desynchronisation of 'clock cells'.

The effect of constant light depends on its intensity. Constant bright light holds the oscillator at a fixed circadian time or 'motionless' state (empty or near empty depending on the exact strength) at Ct 18. Constant dim light may permit continued oscillation but with a lower amplitude and different periodicity on another part of the phase plane (Pavlidis, 1968).

(c) *Van der Pol Oscillators: Kronauer and Wever*

Although used successfully by Wever (1965) and then Kronauer et al. (1982) in models for human circadian rhythmicity, either as a single oscillator or coupled populations, there seems to be no evidence that van der Pol oscillators have been used as a basis of models for insect species.

In van der Pol oscillator models period, stiffness and perturbation from the outside world can be prescribed. However none of the elements of the model represent any real identified component or molecular entity. They have nevertheless been of value, especially when coupled as X and Y systems in accounting for the dissociation and desynchronisation behaviour of human rhythms in constant conditions.

The effect of light can be incorporated into the equation and model oscillators can be coupled together (see sections on population models). When displayed using the threshold concept, actograms of human activity can be simulated realistically. Again this model does not

allow for the presentation of the molecular or physical components of the circadian system which have been identified over the last decade.

(d) Non Linear Differential Equations: Pavlidis

In another purely mathematical approach to modelling, Pavlidis used non-linear differential equations in which light (Pavlidis, 1968) and temperature (Pavlidis et al., 1968) effects could be incorporated by adding or subtracting values.

$$\frac{dr}{dt} = f(r,s) - K.L, \quad (1)$$

$$\frac{ds}{dt} = g(r,s), \quad (2)$$

where r and s are state variables which can take only positive values and, since they may represent something physical, their values lie between limits and the functions $f(r,s)$ and $g(r,s)$ should be of such a form that for $L = 0$ the systems of equations (1) and (2) presents a stable limit cycle which includes part of the s axis (see Fig. 7.1). r is never negative as it may represent the concentration of some substance. s on the same grounds may not be negative. K is a sensitivity coefficient and L represents light intensity.

Light has opposite effects depending on whether the subject is nocturnal or diurnal. Light damps the oscillator in nocturnal organisms, while a lack of light has a similar effect on diurnal organisms (Pavlidis, 1968).

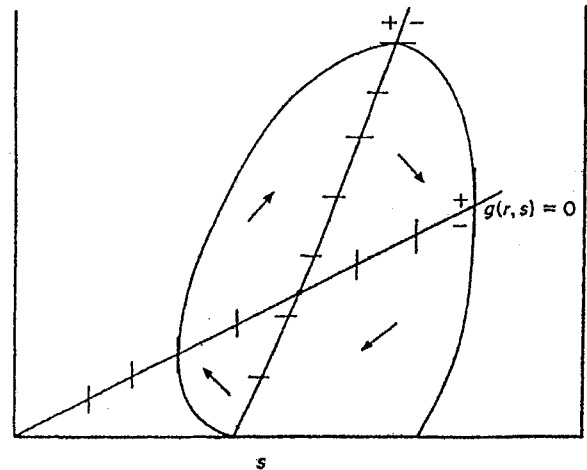


Fig. 7.1. Limit Cycles of the system of equations (1) and (2). From Pavlidis et al., 1968.

The following assumptions were made:

(1) That the total system consists of a primary sub-system (a pacemaker) that is a self-sustained oscillator and which drives a secondary subsystem (a slave; although not necessarily an oscillator), the latter being directly responsible for eclosion (see Chapter 3). The circadian time (Ct) of pupal eclosion is at the phase where the response curve moves from delays to advances (Fig. 3.17).

(2) That the steady state phase shift occurs immediately or in a fraction of a cycle in the pacemaker and the observed transients are due to the slave.

Under these two assumptions one can visualise a very general mathematical model. Thus, a dynamical system which presents a limit cycle (with one degree of freedom) and whose changes of state because of a light stimulus are of very short duration occur almost immediately (See Fig. 7.1). So, if light falls at Ct 16 the system moves immediately at Ct 8 (ie a delay phase shift, $-\Delta\phi$ of 8 h). Light has no effect when it falls between Ct 4 and 12 (the 'dead zone'). The model is topological: if light is on at Ct 18 then the system stays there until its removal.

2. Control systems models for insect circadian clocks

The mathematical oscillator models described in the previous sections say nothing of the forces generating the oscillations essential for the functioning of insect clocks. We need to know what regulates the rhythmic synthesis of the protein, and how the system responds to light and temperature perturbations.

Living systems are replete with homeostatic mechanisms and feedback loops which maintain the constancy of the body, or more realistically, the flow of changes that take place in regular cycles. These systems depend on negative feedback in which the difference between the current situation and a set point, or reference value, regulates the response of the system.

The control systems approach adopted by Goodwin (1965), Johnsson and Kalsson (1972), Gander and Lewis (1979), Ruoff (1999), Leloup et al. (1999), Lewis, Warman and Saunders (1997) envisages and quantifies the interactions between clock components which to a greater or lesser extent correspond to known molecular components as they are currently understood. For example, Hardin et al. (1990) present their molecular mechanism as a simple diagrammatic molecular model. In particular they acknowledge the roles of time delay and feedback in the control of biological oscillators. The integration of the rapidly developing molecular models with the systems models represented by the second group of authors will eventually lead to the fuller understanding of the nature of circadian systems of insects and other organisms.

These living control systems are analogous to the man-made control systems of engineers and can be explained by the same control systems mechanisms. It may be possible therefore to use engineering techniques to understand living systems (see for example, Goodwin, 1965; Kalmus and Wigglesworth, 1960).

In a control systems model, detailed knowledge of the precise physical nature of the components is not essential to an understanding of the overall control mechanism. Only the input-output functions of components, and their interactions need to be specified. A control systems approach to the modelling of circadian clocks is therefore advantageous, firstly since no one clock mechanism has yet been completely elucidated, and secondly in view of the emphasis on the diversity of clock mechanisms in different organisms. The behaviour of components in a successful control systems model should provide information to aid

identification of the physical components involved in the real system. In particular, such information should help to discriminate between processes which are an integral part of the time-keeping mechanism and those which are only driven by the clock, because they exert no feedback on it (Gander and Lewis, 1979).

The concept of feedback has been incorporated into a number of published models of circadian clocks. Sweeney (1974) derived a model from the *Acetabularia* rhythm paradoxes, in which she proposed that circadian rhythms are generated by a feedback mechanism. The active transport into organelles is dependent on the distribution of the molecule being transported. Active transport stops when a critical concentration is reached within an organelle, and passive diffusion re-establishes an even distribution between nucleus and cytoplasm.

The membrane model for circadian clocks incorporates ions and membrane-bound ion transport elements within a biochemical clock (Njus et al., 1974). Feedback arises from the effect of ion concentrations on the activities of the ion transport structures, which in turn affect the ion distributions.

An idea incorporating a reference has been put forward by Benson and Jacklet (1977) who offered a feedback-relaxation oscillator model for the control of the circadian rhythm of compound action potentials as recorded from the optic nerve of the marine mollusc *Aplysia*. In this model a protein is synthesised and its concentration compared with a reference level; if the reference is exceeded then synthesis is switched off, and resumes as the concentration falls below threshold. Cornelius and Rensing (1982) and Drescher et al. (1982) also incorporate feedback in their clock models.

Although the concepts of feedback, references, protein synthesis and diffusion of proteins through membranes have been developed in circadian clock models by a number of authors as described above, only the feedback model for the petal rhythm of *Kalanchoë blossfeldiana* (Johnsson and Karlsson, 1972) is quantitative and predictive. This model was adapted to account for circadian locomotor rhythms of the New Zealand weta *Hemideina thoracica* (Gander and Lewis, 1979).

A feedback model for *Hemideina* has been described as follows. An oscillation in an activity promoting chemical (c) is postulated to control the timing of the active phase of a locomotor rhythm as its concentration passes through a threshold value. The time-delayed concentration of c is compared with a reference concentration (c_{ref}) to generate an error signal, which if positive activates synthesis of c .

The synthesis function is temperature sensitive with a temperature coefficient of 2.0. The rate of loss of chemical from the system is concentration dependent but temperature insensitive. The system oscillates as the result of time-delayed negative feedback. The concentration c' , which is compared with c_{ref} , represents the concentration of c which was in the system a fixed time interval earlier.

The control systems diagram for the single oscillator feedback model is displayed in Fig. 7.2 (Christensen et al., 1984). A large number of combinations of parameter values could be used in the investigation of model behaviour. To reduce the number to manageable size, c_{ref} is retained at a fixed level, and the loss function simplified to a direct proportion of concentration. The period of the oscillation is largely dependent on the time-delay. With a short time-delay the oscillation damps out after a few cycles, and thereafter the period of the oscillation is three to four times longer than the delay. In our simulations the time delay is set so that one iteration represents 20 minutes of real time; so one day of activity is made up of 72 calculations of c .

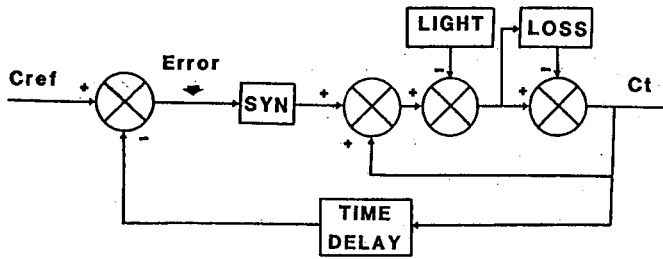


Fig. 7.2. Control systems diagram of the single oscillator feedback model (From Lewis, 1994).

The *Hemideina* circadian clock is sensitive to light. The introduction of light effects in the model is achieved by subtracting values of the instantaneous concentration of the oscillating chemical at the times of 'lights on' at the position indicated in the control systems diagram. Note that this is on the same side of the time delay as ct since the *Hemideina* rhythm responds immediately to light perturbations. For example, activity ceases during prolonged exposure to bright light (above 1 lux), but resumes within about 1 hour of 'light off' (Lewis, 1976). The numerical values for light have no exact relationship with intensity, but the model assumes that light has an intensity dependent effect, at least up to a threshold.

The shape of the synthesis curve also has a critical effect on the behaviour of the single oscillator, in particular in relation to its waveform and response to dim light (Fig. 7.3). To account for the responses of our subjects to dim light, it is necessary to utilise a synthesis function with a relatively steep linear section, and a low upper bound. This at normal temperature accurately predicts the Aschoff's Rule effect shown by the real experimental data (see Chapter 2), and results in an unperturbed oscillation with a saw-tooth waveform.

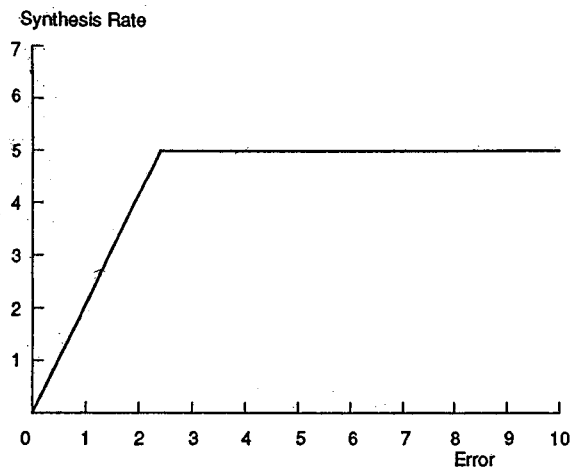


Fig. 7.3. The relationship between synthesis and error in the *Hemideina* feedback model (From Lewis, 1994).

This oscillation is robust and does not exhibit amplitude or phase transients after perturbation (see Christensen et al., 1984). The synthesis component in functional terms represents protein synthesis, which is generally accepted to be an important part of circadian mechanisms. To account for the known temperature responses of the *Hemideina* rhythm, the synthesis function is temperature sensitive, with a temperature coefficient of 2.0. This is achieved by adjusting both the slope of the linear section, and the upper bound according to temperature (Fig. 7.3).

The loss component represents diffusion and loss of an essential activity promoting chemical (protein) through a membrane, and is proposed to be temperature independent (see Njus et al., 1974).

In this single oscillator model of the *Hemideina* clock, the output of the hypothetical underlying clock system is an oscillation in the concentration of an activity-promoting chemical. The overt rhythm recorded in laboratory experiments is displayed as a series of actograms of locomotor activity. These as a rule provide information on the times of activity onsets and ends, but give very little appreciation of the amplitude or waveform of the rhythm.

(a) *The threshold concept*

The threshold concept (Wever, 1965; Aschoff et al., 1971) provides the simplest relationship between the endogenous oscillator and the locomotor rhythm. It proposes that activity is switched on as the concentration of the activity-controlling chemical rises through a horizontal threshold, and switched off as it declines below it. It is tempting to elaborate this idea with the proposition that the form of the active phase reflects the area of the oscillation above threshold. The alternative hypothesis, that activity is triggered as the concentration of the critical chemical falls below a threshold, is not supported by experiments on the responses of the *Hemideina* rhythm to temperature perturbations (Gander, 1979).

(b) *Tests of the model*

One of the major requirements of a model is to predict the behaviour of the modelled system under novel experimental conditions, and to test the predictions against new observations. However, early in the development of any model, the main task is to account for the existing data or known information of the system under study.

(c) *General properties of the model*

When run on a digital computer, with a fixed reference value and loss rate, the control systems model described above exhibits self-sustained, temperature-compensated oscillations around the reference level with a variety of combinations of different parameter values. The following generalisations can be made about the behaviour of the system (Gander and Lewis, 1979):

1. Increasing the *synthesis rate* increases the stability of the oscillation. With all other parameter values remaining fixed, decreasing the synthesis rate to below a critical minimum value results in damped oscillations, while increasing it above the level necessary for self-sustained oscillations increases the amplitude but has only minimal effect on the period of the cycle.

2. Increasing the length of the *time delay* increases the stability of oscillations and the period of the rhythm. There is a critical minimum length of time delay necessary for the system to develop self-sustained oscillations; below this the oscillations damp out.

(d) Light responses

The *Hemideina* circadian clock is extremely sensitive to light. Activity ceases in constant light of intensity greater than 1.0 lux, and resumes about one hour after the return to darkness. In constant dim light (LL) (intensity about 0.1 lux) the free-running period is longer than in constant darkness (DD), consistent with Aschoff's Rule for dark-active animals (Lewis, 1976).

Assuming that light has an intensity-dependent effect up to a threshold, the model simulates all the known responses of *Hemideina* to light at different intensities. In simulated constant dim light, the oscillation continues, but at a lower mean level and with a longer period than in constant darkness. It therefore mimics the insect clock in supporting Aschoff's Rule. In simulated continuous bright light (Fig. 7.4) the model oscillation drops below threshold and damps out; however it rises rapidly back through the threshold on the return to constant darkness. This is in agreement with the previously noted inactivity of *Hemideina* during prolonged bright light and the rapid resumption of activity after 'light off' (Gander and Lewis, 1979).

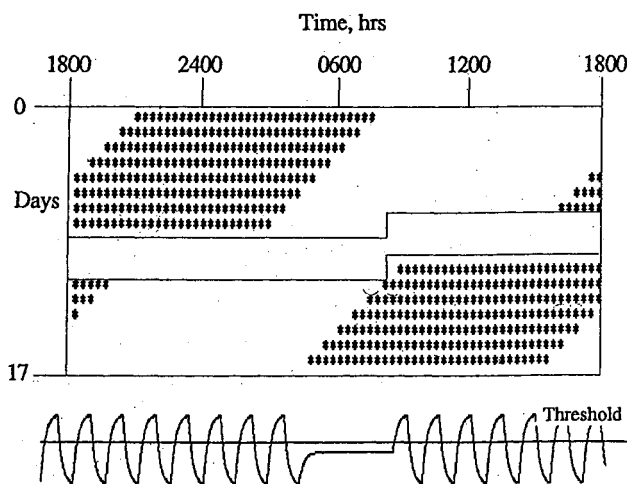


Fig. 7.4. Simulation of the effect of constant bright light on the rhythm. The light is on at the times between the horizontal bars (From Lewis, 1994).

The model oscillator is entrained by light cycles, and takes up the appropriate phase relationship with the light. Single pulses of light advance or delay the simulated rhythm (e.g. Fig. 7.5) depending on the circadian time of administration, and the phase response curves (PRCs) of simulated data correspond well with the animal results. (See Fig. 7.6, in which the solid lines trace the PRCs of simulated phase changes for 8 h and 12 h pulses). Fig. 7.7 illustrates a simulation of the effect of a single 4 h pulse falling near Ct 1 and destroying locomotor activity for several days. Examination of the wave-form of the oscillator suggests

that the pulse may have pushed the oscillator towards its singularity (Winfree, 1971) and held it below threshold. Single 8 h and 12 hour pulses falling at the same Ct also severely disrupt the locomotor rhythms of *Hemideina*, but this may be attributed to the desynchronisation of a population of oscillators; this point is discussed in a later section.

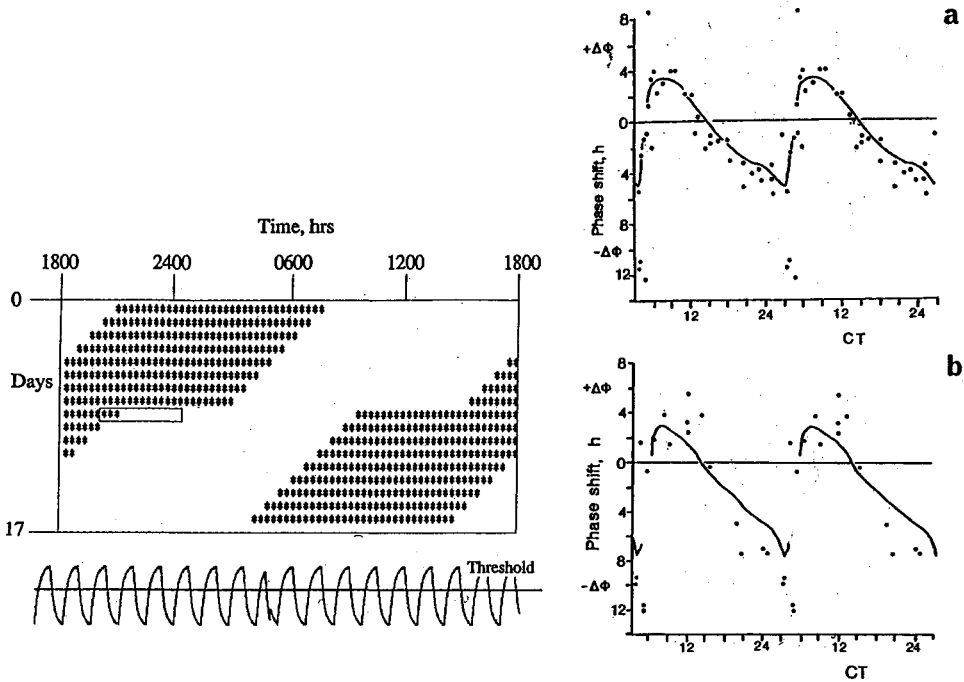


Fig. 7.5 (left). The simulation of a phase advance of a rhythm by a 4 h light pulse (open box) falling after the midpoint of activity. Note the perturbation of the waveform by the pulse (From Lewis, 1994).

Fig. 7.6 (right). Phase response curves (PRCs) for (a) 8 h pulses, and (b) 12 h pulses of light in *Hemideina*. The dots are experimental data; the solid lines are simulated results (From Lewis, 1990).

(e) Temperature responses

Although invertebrate clocks may be entrained and phase-set by temperature changes within the normal range of biologically relevant temperatures, the periods of circadian rhythms are more or less independent of temperature, suggesting a high degree of temperature compensation of the rate-determining processes (Pittendrigh, 1974) (see Chapters 2 and 3). Temperature compensation of circadian clocks is defined as one of their basic properties, and may be seen - in the invertebrates at least - as an adaptation to maintain their periods within the range of entrainment by the natural agents. In temperatures below those normally encountered in nature, circadian clocks of poikilotherms generally stop, or at least the overt activity becomes arrhythmic (Gander, 1979).

The free-running period, as measured as the mean time from one onset of activity to the next, changes very little over the range of constant temperatures (15°C to 30°C); the

temperature coefficient is close to unity. For *Hemideina* the mean value is 0.99 ± 0.07 derived from 66 estimates of temperature coefficients over the range 15°C to 25°C.

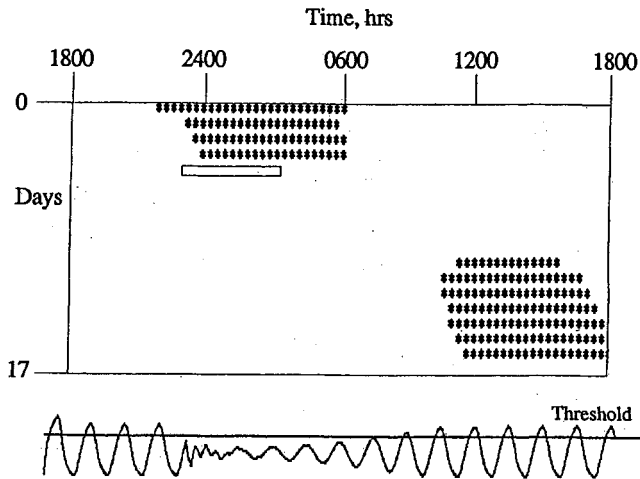


Fig. 7.7. The simulation of the destruction of a rhythm by a single 4 h light pulse (open box) (From Lewis, 1994).

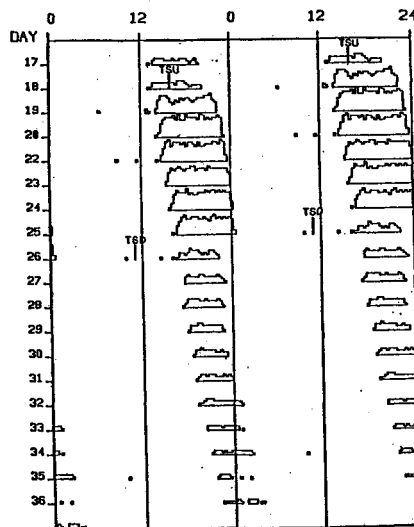


Fig. 7.8. Part of a double-plotted *Hemideina* locomotor rhythm illustrating the effects of a temperature step-up from 20° to 25°C on day 18, and a temperature step-down on day 26 (From Lewis, 1994).

The relationship between temperature coefficient of the clock and temperature measured over this range of temperatures is non-monotonic with a minimum period at about

20°C. Furthermore the amplitude and active phase length of at least some rhythms show increased variation with temperature. Some records show increased active phase lengths and amplitude with increasing temperature (e.g. Fig. 7.8) (Gander, 1979). Below about 15°C activity ceases, and resumes a few hours after a rise in temperature (Gander, 1979). This pattern of behaviour in response to low temperature suggests that the internal circadian clock is stopped, or at least the oscillation is damped, in the cold. The clock is not independent of temperature in the range 15°C to 30°C as it is entrained by square-wave temperature cycles (12 hours warm:12 hours cool) of 6°C or greater amplitude. The phase relationship between the activity and a 24 hour temperature cycle is a function of the free-running period of the rhythm, with activity onsets occurring some time after the fall in temperature (Gander, 1979). It is clear that the simulation of temperature compensation is one of the major challenges in the modelling of circadian mechanisms.

Temperature is postulated to affect the proposed circadian oscillator mechanism by altering the rate of synthesis of the activity promoting chemical (*c*) (temperature coefficient of 2.0). In addition, the activity rhythm in LL (0.1 lux) at 16°C was sometimes destroyed for the duration of the dim light exposures. When the rhythm persisted the period increases were large and the amplitude or extent of the active phases reduced (Christensen et al., 1984).

Simulations of the effect of temperature on the period and amplitude of the *Hemideina* rhythm demonstrate that the model, when configured as described above, accounts for both the conservation of period and the changes in extent of activity over the normal range of temperatures. Examination of the waveforms of the hypothetical oscillations provides clues to the mechanism of compensation since the amplitudes of oscillations at elevated temperatures are greater than at the low temperatures. This means that whilst the rate of increase in the synthesis of the essential chemical is greater at high temperature, the loss phase begins with a higher concentration so takes longer, hence the overall period is not greatly different from that at the lower temperatures. What the system gains in speed by the accelerated synthesis rate, it loses by the protracted diffusion phase. The increased amplitude of the oscillation at high temperature can lead to a longer active phase and perhaps greater amplitude of extent of activity.

The simulation of entrainment of the locomotor rhythm with temperature cycles and the responses to pulses and steps, must go hand in hand with temperature compensation, since the same model with the same set of parameter values must account for all these facets of temperature responses. *Hemideina* locomotor rhythms are entrained by square-wave temperature cycles of 6°C or greater amplitude; the phase relationship between the rhythm and the 24 hour entraining cycle is dependent on the period of the rhythm. Generally, activity begins shortly after the onset of the cool phase, as we expect for a nocturnal animal (Gander and Lewis, 1979). Similar entrainment in simulated square-wave temperature cycles of 6°C amplitude or greater is exhibited by the model oscillation (Gander, 1979).

C. THE CLOCK AS POPULATIONS OF OSCILLATORS

So far, the insect circadian system has been modelled as a single oscillator, equivalent perhaps in structural terms to a single cell or organelle. This level of model accounts for many of the basic properties of insect circadian rhythms, including light and temperature entrainment, responses to single long or short pulses, and temperature compensation. It is also clear that in a multi-cellular organism there must be more than one unit to safeguard against loss or death of cells.

1. 'Free-run period lability'

A single oscillator model also leaves unexplained a class of behaviour observed in a variety of organisms and labelled 'free-run period lability' by Pavlidis (1969; 1978a). This category of rhythmic behaviour includes rhythm splitting, spontaneous changes in free-running period, desynchrony of the rhythm, and after-effects of a range of pre-treatments (see Pittendrigh, 1974; Chapter 6).

(a) Rhythm splitting

Rhythm splitting may be evident in an overt locomotor rhythm in a number of ways. One of the classical examples is the subdivision of the locomotor rhythm of the tree-shrew, *Tupaia belangeri* into two, or in some cases three, parallel components in response to a change in light intensity (Hoffmann, 1971). Another example of the same behaviour is rhythm splitting in *Hemideina* (Christensen and Lewis, 1982) following a critically placed light pulse, and a similar phenomenon has been induced in the cockroach *Leucophaea maderae* by the injection of the drug azadirachtin into a free-running animal (Han, 1986).

In other examples of rhythm splitting the components do not run in parallel, but diverge and converge at different periodicities, with a small degree of mutual interaction. The stridulation rhythm of male crickets *Teleogryllus commodus* occasionally splits in this manner (Wiedenmann, 1983), and the *Hemideina* free-running records reveal splitting patterns of this sort (see Fig. 7.9).

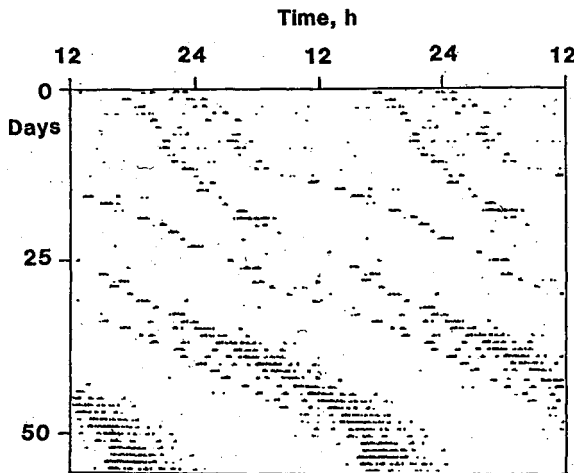


Fig. 7.9. Rhythm splitting in *Hemideina thoracica* with components of different periodicities (From King, 1988).

(b) Spontaneous changes in period

Spontaneous changes in period of free-running rhythms are quite normal in a wide variety of animals; indeed it is remarkable if a rhythm retains a constant period over a long time (Pittendrigh, 1974). The period changes may appear in some cases to be random (e.g. Christensen and Lewis, 1982), whereas others systematically increase in period after the first ten days or so in constant conditions (Fig. 7.10). Others describe patterns of shortening and lengthening of period and active phase length for many days displaying recognisable patterns of 'scalloping' (Lewis et al., 1991).

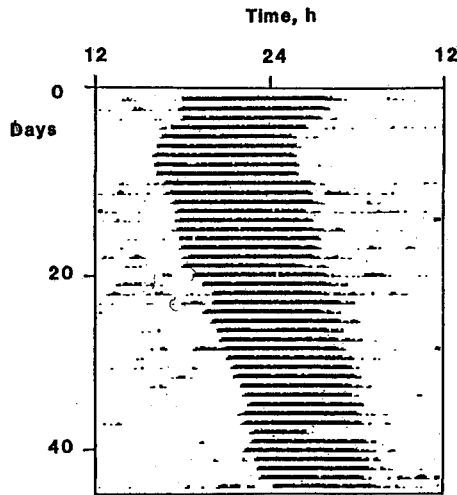


Fig. 7.10. Actogram of the free-running locomotor rhythm of *Hemideina thoracica* in DD at 20°C showing the spontaneous increase in period after about six days of free-run (From Lewis, 1994).

(c) After-effects

After-effects are defined as changes in free-running period immediately following particular types of pre-treatment, especially single pulses, dim light and entrainment (Pittendrigh, 1974). These after-effects are particularly clear in *Hemideina* following entrainment by long period light cycles (e.g. LD 8:23) (Fig. 7.11) (Christensen and Lewis, 1982). Typically the free-running period following entrainment to long-period light cycles is significantly longer before the period finally returns to close to its pre-entrainment value.

(d) Rhythm shattering

Rhythms may shatter spontaneously (Fig. 7.12), or may be forced to shatter by a single perturbation as illustrated in Fig. 6.4. The effect of a pulse of dim red light falling at a critical time is to shatter the locomotor rhythm into a number of short active phases. These components can either reform spontaneously, or as in the figure or be brought back into synchrony by entrainment (see Fig. 6.4). The circadian time of the pulse at which this shattering occurs is about the time that large phase delays become advances (see the PRC for

12 hour light pulses, Fig. 7.6). The timing of the pulse is critical, since the second pulse (arrow 2) administered at about the same circadian time produced a zero phase change.

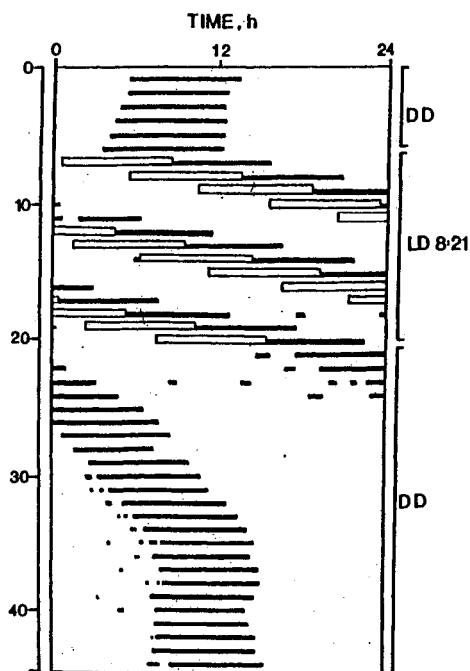


Fig. 7.11. After-effects of long-period light entrainment of *Hemideina thoracica*. The open boxes are the light pulses (LD 8:21); DD indicates the times of total darkness (From Lewis, 1994).

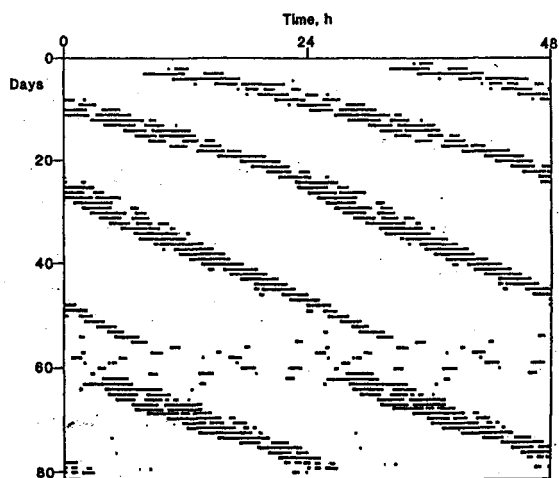


Fig. 7.12. Double-plotted *Hemideina* rhythm demonstrating spontaneous shattering and recovery of rhythmicity (From Lewis, 1994).

2. Modelling population behaviour

Many of these observations could be simulated with single oscillator models by the addition of random fluctuating parameters or numerous extra parameters (Pavlidis, 1971a, 1978a). They can however be accounted for more simply if the clock is assumed to consist of a population of interacting oscillators (e.g. Winfree, 1967; Pavlidis, 1971a, 1978b; Pittendrigh, 1974; Berde, 1976; Pittendrigh and Daan, 1976; Daan and Berde, 1978; Enright, 1980).

A range of population models with varying degrees of complexity can be hypothesised. The simplest is a single population of loosely linked units with their natural periods distributed unimodally. The basic *Hemideina* population model (Christensen and Lewis, 1983) consists of a unimodal population of up to 30 feedback oscillators with periods ranging from 18 to 32 hours (mean 25.5 hours), and similar amplitudes (Fig. 7.13). Coupling between unit oscillators is weak and achieved by partial sharing of the feedback information. A portion of the feedback information comes from the time-delayed value of each individual, the remainder from the mean of the pooled time-delayed values. Thus with strong coupling (e.g. 0.8 on a scale of 0 to 1.0) the majority of the feedback information comes from the pool, and little from the individual; with low coupling, the majority is from the individual. For computational simplicity the coupling between all individuals is the same.

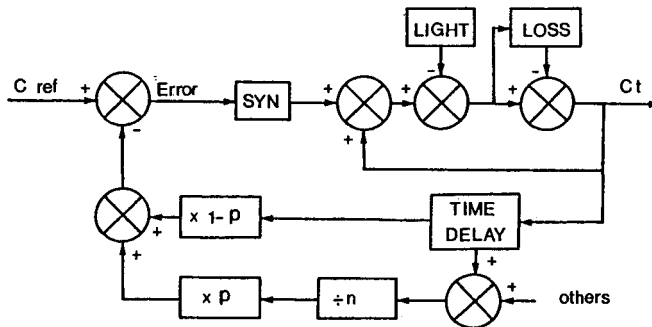


Fig. 7.13. Control systems diagram of the population model for the circadian system of *Hemideina thoracica* (From Lewis, 1994).

As in the single oscillator model, light is proposed to have an immediate effect on the population, and continues for the duration of the pulse without adaptation. 'Light on' is simulated by subtraction of a value (related to intensity) from the concentrations of the all the individuals equally.

In the single oscillator model, the active phases are triggered as the activity-promoting chemical rises above threshold. This concept has to be modified in the population model, so that activity depends on the number of individual oscillators above threshold. In addition to the display of the combined activity of the population, the behaviour and waveform of each individual oscillator may be examined to provide further insight into the interactions of the group.

Simulations of *Hemideina* locomotor rhythms with this simple population model consisting of either 20 or 30 units are performed using similar parameter values for the synthesis and loss functions as in the single oscillator version. The time-delays of the units are varied to give the required range and distribution of natural periods.

The strength of the coupling factor has a paramount effect on the behaviour of the rhythm, and 'realistic' rhythms can be achieved over a relatively narrow range of coupling factors. At high values (above 0.5) all the oscillators are mutually entrained and act as one; with low coupling (below 0.3) the number of linked oscillators is very low leading to arrhythmic behaviour. With intermediate values spontaneous changes in period and breakdown and recovery of rhythms can be simulated (Fig. 7.14), so matching the behaviour of the real circadian system.

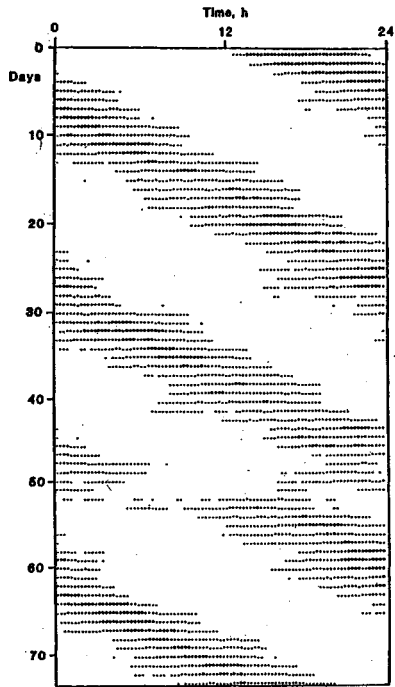


Fig. 7.14. Simulation of spontaneous shattering and recovery of a free-running *Hemideina* rhythm using the population model (From Lewis, 1994).

The simulated rhythm can also be shattered by a critically timed pulse of light (Fig. 7.15). Examination of the behaviour of each of the individual oscillators confirms that they remain at full amplitude, but out of synchrony with each other. The desynchronisation of the rhythm is brought about by the different responses of the individual oscillators to the pulse, falling for some at the circadian times that led to advances, and for others at the times for delays. Members of the population are therefore torn apart by their divergent behaviour. Resynchrony may come about as the result of an increasing number of individuals falling into

line and thus creating a nucleus for mutual entrainment. Alternatively a major perturbation resynchronises the population.

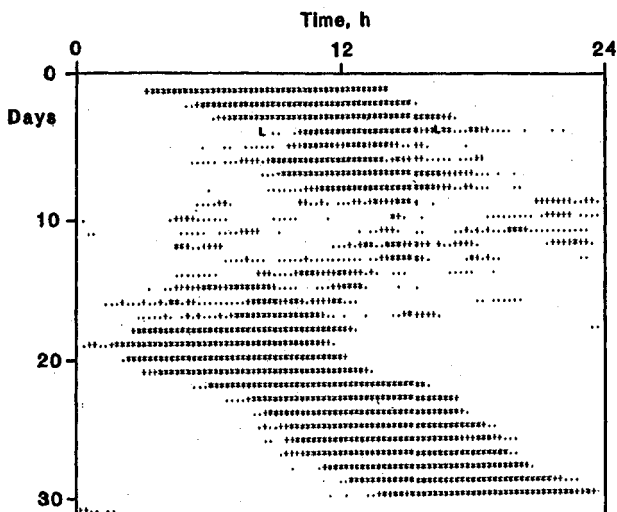


Fig. 7.15. Simulation of the breakdown of a free-running *Hemideina* rhythm by a single 8 h light pulse (L – L on day 4) using the population of feedback oscillator model (From Lewis, 1994).

This explanation for desynchrony in the *Hemideina* rhythm contrasts with the singularity phenomenon proposed as the explanation for arrhythmicity induced by single perturbations in the *Drosophila* eclosion rhythm (Winfree, 1971), the *Gonyaulax* rhythm of bioluminescence (Taylor et al., 1982) and the *Kalanchoë* petal rhythm (Englemann and Johnsson 1978) (see Chapter 3). In these examples arrhythmicity is seen as the result of damping of each of the oscillators in the system.

It is interesting however that the single oscillator clock model for *Hemideina* can be forced towards the singularity at least for a short time by a single light pulse of critical strength and timing (Fig. 7.7), but in view of the persuasive evidence for coupled populations of oscillators as the basis of the clock system of this insect, more emphasis is placed on the desynchronisation hypothesis as an explanation of arrhythmicity in this species (Christensen and Lewis, 1982).

A further clear test of the population model is the simulation of after-effects, particularly entrainment after-effects, which are very pronounced in this insect (Christensen and Lewis, 1982). A population of 30 loosely linked oscillators can be shown to exhibit after-effects following 20 cycles of simulated long-period light cycles (LD 12:20) (Christensen and Lewis, 1983), although they do not persist for as long as in the real data. Analysis of the behaviour of the individual oscillators during entrainment reveals that the normally unentrained long-period oscillators are forced into synchronisation with the main group. They continue to be synchronised for a while after entrainment and exert their influence by lengthening the overall period of the main group for several cycles before they break away to once again to fall out of synchrony. The output of the synchronised population then returns to normal.

The progression from the single to multi-unit circadian clock model for *Hemideina* gives greater understanding of the nature of this insect's clock and is physically realistic.

(a) Redundancy of oscillators?

The single unimodal population model for the circadian clock of *Hemideina* adds significantly to the understanding of the nature of the clock. However, it is not anatomically realistic, since investigations on the location of the pacemaker of this insect support the view that a clock control centre (pacemaker) resides in each optic lobe (Lewis and Waddell, 1987), as is the case for several other exopterygote insects (Brady, 1982; see Chapter 8). The current perception is that a population of oscillators is located within each optic lobe. The removal of both optic lobes destroys the locomotor rhythm, but the rhythm continues following single lobectomies, generally with increased period. The two clock centres therefore seem to be equivalent or redundant. They are linked strongly together, since in the majority of free-runs the active phases are not split, and there is no evidence for the same kind of bimodality as has been recorded for the ERG rhythm of *Blaps gigas* (Koehler and Fleissner, 1978; see Chapter 6). At this stage, therefore, it seems that in functional terms a single unimodal population of oscillators is adequate to account for the behaviour of the two, physically separated clock centres in the optic lobes.

3. Insect clocks as coupled X - Y oscillators

Many of the basic properties of the circadian system of *Hemideina*, such as temperature compensation and entrainment, the responses to dim and bright light, and phase control by light pulses, have been accounted for by a single oscillator feedback model (Gander and Lewis, 1979). In addition, some of the more complex free-run lability is explained by a model comprising a single population of loosely coupled feedback oscillators (Christensen and Lewis, 1983).

In this section the model is extended to cover these features by the introduction of a second population of feedback oscillators which interacts with the original to produce the previously unaccounted for characteristics. The concept of two control centres for regulation of insect rhythms is not new. Pittendrigh (1960) proposed the A and B (pacemaker/slave) model for the control of *Drosophila* eclosion rhythms (see Chapter 3), and more recently further reports of simulations of such pacemaker/slave relationships have been published (Pittendrigh, 1981).

An alternative approach to the A and B model derives from the analysis of the circadian flight activity rhythms of the mosquito, *Culiseta incidens* (Clifton, 1984; Chapter 2). These rhythms exhibit a variety of types of free-run lability, including some of the very few examples of circa-bi-dian locomotor rhythms. Analysis of these rhythms has led to the development of a dual pacemaker model consisting of a labile evening oscillator (E) mutually coupled to a stable morning oscillator (M). The E and M oscillators run with different periods and account for the free-run lability (including the very long-period rhythms) evident in the actograms. In this dual pacemaker model the coupling is mutual, and each component (E and M) contributes to promotion of activity. This contrasts with the pacemaker-slave (A and B oscillator) concept proposed by Pittendrigh (1960, 1981) for the adult emergence rhythms of *Drosophila* in which the coupling is unilateral from A to B. Clifton also introduces a slave oscillator to explain some of the complexities of his data. A similar concept of E and M pacemakers is also supported by the circadian flight-activity rhythms of another mosquito, *Culex pipiens quinquefasciatus*, which also have two major overt components (Jones, 1982). The *Hemideina*

rhythms show no evidence for evening and morning components, and we therefore feel that the E and M models described above are not appropriate. The scalloping patterns evident in so many records indicate interaction between components and this rules out the concept of unilateral coupling proposed in the *Drosophila* A and B oscillator model.

It is proposed that the circadian control system for the locomotor rhythmicity of *Hemideina* also comprises two major centres (X and Y), each consisting of a population of linked oscillators having a diversity of native periods and characteristics. These populations in free-running conditions interact mutually to produce the variety of free-run lability evident in numerous actograms of *Hemideina* locomotor activity rhythms. In contrast to the E and M model published for the two species of mosquito, only one population (Y) is directly involved in the regulation of locomotor activity; it is the interaction of the two pacemakers that creates the lability found in free-running *Hemideina* rhythms.

In overall concept this model most closely resembles the human dual pacemaker model of Kronauer et al. (1982) in which the Y component regulates the sleep-wake pattern and the X component the core body temperature. It is significantly different from the A and B models for the *Drosophila* eclosion rhythm, and the E and M model for the flight rhythms of mosquitoes, and is novel in treating each pacemaker as a linked population of oscillators.

(a) *X - Y characteristics of free-running rhythms*

The following features are frequently exhibited in actograms and support the X-Y model.

1. Scalloping is characterised by the systematic decrease and increase of circadian period together with compression and expansion of active phases at about 16-day intervals (e.g. Fig. 7.16). The active phases are rarely totally lost, although there is frequently reduction in their coherence. Scalloping is identified in 28 per cent of the samples, and generally associated with circadian rhythms with periods greater than 24 hours. The mean period of the scallop rhythm is 16 days; the range from 8 to 36 days. A line (the X line, see model section) has been added to Fig. 7.16 joining successive phase points of the scallop rhythm to indicate the average pathway of a second major component (having a period of 22.7 hours in this example). The phase point is taken as the start of the active phase of the day before the increase in circadian period during each of the scallop cycles.

2. 'Day-skipping' is the systematic loss and recovery of the active phase, and when repeated it results in more or less regular blocks of activity. This label has been taken from the terminology developed to summarise the free-running flight rhythms of mosquitoes (Clopton, 1984). In *Hemideina* (e.g. Fig. 7.17) the active phases in the days preceding day-skipping gradually reduce in length until activity ceases, to be followed a few days later by a steady increase in locomotor activity, often phase-advanced on the original.

3. Spontaneous change from a short period (often close to or less than 24 hours) to a long (greater than 24 hours) period rhythms during the first 20 days (mean 9.3 days) in DD following natural entrainment (e.g. Fig. 10). Half of the actograms analysed show this type of period increase. The mean period of a sub-set of 50 examples during the days before the change is 24.2 hours and after 25.14 hours, giving a mean increase of 0.94 hours (maximum single increase of 3.5 hours). In contrast the active phase length reduces after the spontaneous change in period from a mean of 8.9 to 7.9 hours.

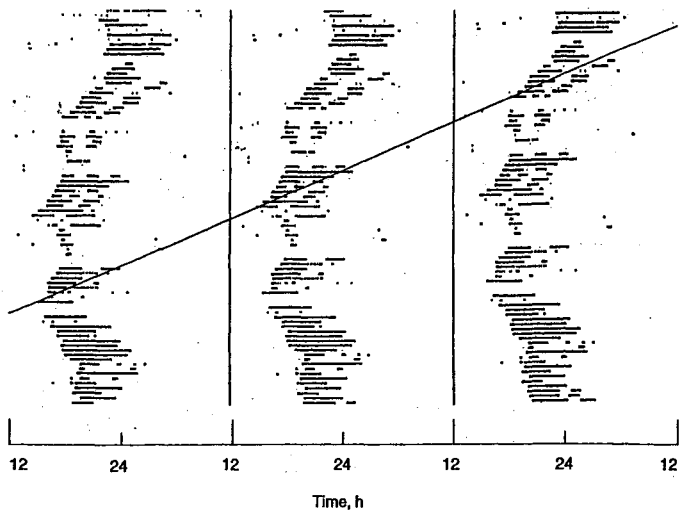


Fig. 7.16. A triple-plotted actogram showing scalloping of the circadian locomotor rhythm in *Hemideina thoracica*. The diagonal line passing through equivalent phase points of the scallop rhythm is the X-line. Its slope reflects the overall period of the X pacemaker (22.7 h in this example). The average period of the four cycles of the scalloped rhythms is about 19 days. See text for more explanation (From Lewis, Bullivant and King, 1991).

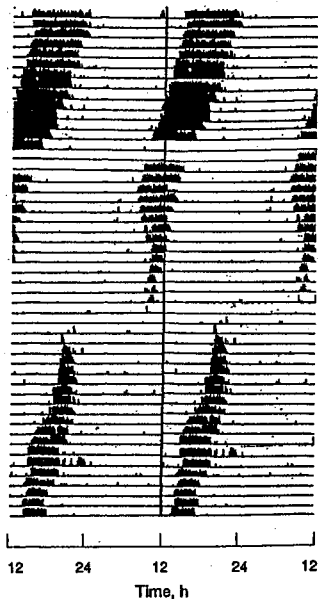


Fig. 7.17. Actogram showing day-skipping in *Hemideina thoracica* (From Lewis, Bullivant and King, 1991).

4. *X - Y models for insect clocks*

(a) *Description of the models*

The analysis of locomotor activity rhythms suggests that the circadian clock of *Hemideina* comprises two mutually linked pacemakers (X and Y), each made up of a population of coupled feedback oscillators. The Y pacemaker has an overall period of about 25 hours, and directly regulates the timing of locomotor activity. The X pacemaker runs faster (period about 23 hours), and has no direct effect on the active phase (Lewis, 1988). The period of the X pacemaker is derived from the X-line superimposed on the scalloped rhythms as in Fig. 7.16. The X and Y pacemakers are loosely linked with unequal positive coupling by the exchange of small fractions of their instantaneous values. The X system has about a fourfold advantage over the Y pacemaker. In constant conditions the pacemakers pull apart, but in field conditions these two pacemakers are kept in synchrony by the natural cycles of light and temperature etc., and the onset of activity is phase-set to dusk.

The variety of patterns of free-running behaviour recorded in constant laboratory conditions stem from the changes in synchrony and phase relationship of these two components in the absence of external entrainment. The variations in behaviour between individual subjects in constant conditions are a consequence of small inter-individual differences in the parameter values of their control systems - such as native periods of the unit oscillators or coupling factors - which may have minimal consequences on the timing of behaviour when the clock is entrained by natural *Zeitgeber* in the field.

The output of the X oscillators does not contribute directly to the locomotor activity, but influences the phasing of activity through the coupling process to the Y system. In the simulated actograms vertical bars display the progress of the X system over time, and its phase relationship with the locomotor rhythm. Each bar indicates the time at which the combined concentrations of the X oscillators reach a peak; parallel bars occur when the combined concentrations fluctuate around the maximum values. Bars are missing when the X system is temporarily damped or oscillates below a critical level of concentration.

Each unit oscillator is a time-delayed negative feedback system controlling a circadian period oscillation in the production of a hypothetical chemical (Gander and Lewis, 1979; Christensen et al., 1984; Lewis, 1990). In both the X and Y populations, the unit oscillators are loosely coupled by partial sharing of their feedback information (Christensen and Lewis, 1983). The members of the Y population are perturbed by light through subtraction of chemical values while light is on in proportion to the light intensity. The X system may also be entrained by light, although the sensitivities of the X and Y oscillators are not necessarily the same.

(b) *Simulations of X - Y behaviour*

The aim of the simulations was to account for the various categories of rhythms described above by relatively small variations in both the coupling within and between pacemakers, and the dominance of the X pacemaker over the Y. Similarities between the simulated and real actograms were taken as evidence of support for the dual pacemaker model, but they do not exclude models of other configurations of oscillators.

Scalloping (for animal results see Fig. 7.16 and simulated data in Fig. 7.18) occurs when the dominance of the X pacemaker is such that the Y pacemaker is generally entrained to it, but at more or less regular intervals breaks away to take up a longer period for a few cycles before

resynchronising to the X. The frequency of the scalloping decreases with increasing dominance of the X system to the point when the Y system is totally entrained to the X.

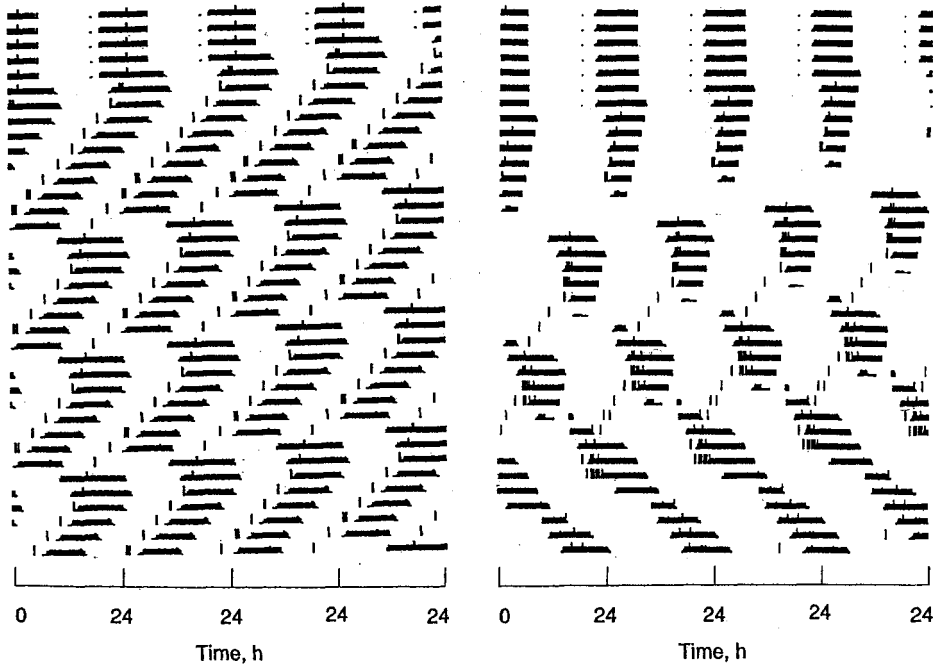


Fig. 7.18 (left). Quadruple plotted simulation of scalloping with the X and Y dual pacemaker model. The horizontal black bars represent active phases, and the vertical bars are the times of the peaks of the combined X oscillators. The rhythms are entrained for the first five days (LD 12:12) and free-run thereafter. The ends of the light pulses are depicted by the dot preceding the onset of activity. The scalloping patterns are produced by the interactions of the two pacemakers (From Lewis, Bullivant and King, 1991)

Fig. 7.19 (right). Simulated day-skipping of a *Hemideina* locomotor rhythm in DD following seven days in LD 12:12. In DD the active phases shorten then cease before resuming after several skipped days at an earlier phase relative to the original activity. The rhythm finally adopts the longer period of the Y pacemaker (From Lewis, Bullivant and King, 1991).

In the simulation of day-skipping (Fig. 7.19) the Y population is relatively strongly coupled, and the two pacemakers never synchronise during the free-run. Their interactions produce large changes in active phase lengths, and times of no activity (day-skipping) when the two pacemakers are completely out of phase. In the example displayed in the figure, the Y pacemaker eventually dominates, and breaks away to run at its own periodicity with minimal interaction with the X pacemaker.

The spontaneous increase in period during the first few days of free-run is simulated in Fig. 7.20. In the days following entrainment, the Y system is synchronised to the shorter period X pacemaker, but eventually breaks away to take up its natural longer period without significant interaction with X. The initial synchrony is seen as an after-effect of entrainment during which the coupling of the X population is enhanced to give dominance over Y. The

cohesion breaks down in the absence of entraining cycles and the dominance over the Y population is lost.

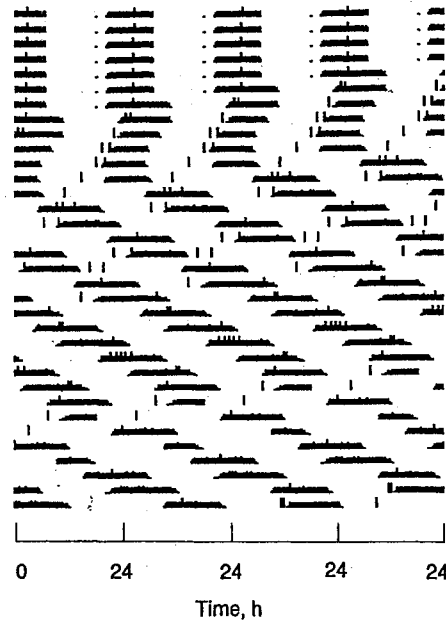


Fig. 7.20. Simulation of the spontaneous increase in the free-running period of a *Hemideina* locomotor rhythm from less than 24 hours during the first six days in DD following light entrainment (LD 12:12) (From Lewis, Bullivant and King, 1991).

DISCUSSION

Free-running locomotor rhythms in *Hemideina thoracica* generally persist for many weeks. The rhythms may be remarkably precise and predictable, or exhibit spontaneous period lability. Our analysis of a large number of rhythms has allowed us to define five major types of lability: scalloping, rhythm splitting, day-skipping, arrhythmicity, and spontaneous change from short to long period. These resemble the spontaneous changes exhibited by the mosquito *Culiseta incidens* (Clopton, 1984), with the exception that circa-bi-dian rhythms are never seen in *Hemideina*.

The behaviour of clocks in constant conditions provides data for the development and testing of models of the nature of the circadian systems. Many of the basic properties of the *Hemideina* clock can be accounted for by a single oscillator feedback model (Gander and Lewis, 1979; Christensen et al., 1984), while aspects of free-run lability are explained by a model incorporating a weakly-coupled population of feedback oscillators (Christensen and Lewis, 1982). However, many of the characteristics revealed in this analysis cannot be explained by these two models.

Visual analysis of *Hemideina* rhythms, especially those illustrating scalloping, indicates two major interacting components in locomotor activity rhythms; one with a short circadian period, and the other with a longer period. We have therefore developed the dual pacemaker (X

- Y) model for *Hemideina*. In this we hypothesise that one pacemaker (the Y, comprising a population of up to 32 coupled feedback oscillators) has direct control of the timing of locomotor activity, and interacts with another similar pacemaker (X) which regulates other physiological functions, such as the circadian rhythm of cuticle deposition (King, 1988). An indication of the overall period of the X population is gained from the X-line which in obviously scalloped rhythms joins defined phase points of the circa 15 day scallop pattern.

This model has similarities to the human circadian model in which two single van der Pol oscillators are linked (Kronauer et al. 1982), but is different from most previously published dual pacemaker models in that both our pacemakers are made up of a number of circadian period oscillators.

We see no evidence for evening and morning components of activity in the actograms, and hence have not favoured the E and M approach taken for both the mosquito circadian system (Clopton, 1984), and a number of vertebrate studies (e.g. Daan and Berde, 1978; Meijer et al., 1990). In our model the control of the timing of locomotor activity is through the output of a single population of loosely coupled oscillators. At times small groups of oscillators from this population break away from the main group and form a minor component running in alongside the main active phases.

The optic lobes of the brain are essential for circadian locomotor rhythms (Waddell et al., 1990; see also Chapter 8), although rhythmicity continues after unilateral lobectomy, generally with increased period. In the present form of the dual pacemaker model we propose that the rhythmic behaviour of the two optic lobes is encompassed by the single Y population; in reality there is coupling between the two groups of cells of the optic lobes.

From the simulations we see that all the categories of free-run lability identified by the analysis of the numerous rhythms can be accounted for by the model on the basis that the computer simulations give output that resembles the real data. We take these successes in matching real behaviour as support for the idea that the concepts underlying the model reflect the essential functioning of the animal system. We recognise that individual *Hemideina* have essentially identical circadian mechanisms, with small inter-individual differences. These differences may have only marginal significance in field behaviour where natural entrainment ensures the adoption of the appropriate phase relationships of activity onsets to sunset. In constant conditions these variations in the parameter values may lead to the variety of lability described in this study. When running the model it is evident that small changes in parameter values give rise to significant differences in computed output. All the behaviour we record can therefore be explained in terms of small differences in the interactions of the major pacemakers.

From the relatively small number of analyses of insect circadian rhythms it seems that whilst all support a very general model of linked pacemakers, there is no consensus when the detail of the models are examined. This may well reflect real differences in the circadian systems of the variety of insects examined, and we would not be prepared at this stage to generalise the *Hemideina* model to a wider group of insect species.

D. GENERAL DISCUSSION

If we were to set aside our computer keyboards and controlled environmental conditions and record patterns of insect behaviour in the field, we would undoubtedly find that the vast majority of species demonstrate regular daily patterns of movement, feeding, migration, hatching and emerging which may be seen to be adaptations to the regular and predictable physical and biotic changes in the environment. The evolutionary reasons for these

daily rhythms seem obvious: to avoid predation or harsh physical conditions, or to optimise predation or migration, and so on. But these vital ecological considerations leave unanswered the mechanistic question of how living organisms control the timing of their patterns of behaviour.

From countless experiments using the traditional experimental methods of recording activity in timeless conditions, it has become undeniably clear that within insects there are endogenous biological clocks whose mechanisms are analogous to man made clocks and timers, and are fashioned by genes out of proteins at the subcellular level of organisation. The adoption of the notion that these are living oscillators with the same characteristics as physical, hydraulic or chemical oscillators allowed for the development of hypotheses and models of how biological clocks work.

There are of course a number of sophisticated mathematical ways of describing such oscillators. The van der Pol oscillators used by Wever (1972) and colleagues, the differential equations of Pavlidis (1968) and the limit cycle description of Winfree (1970) have all in their particular ways been successful in extending our understanding of the nature of biological clocks. The feedback models used here to extend the understanding of the circadian clock of *Hemideina*, are based on the control systems approaches of Johnsson and Karlsson (1972). In some ways they are less sophisticated than the other quantitative models, but have the advantage that even in the simplest presentation each of their components may be matched with some feature of the molecular physiology of the timing system. In an early attempt to bring together the two seemingly divergent approaches to the understanding of the nature of biological clocks, Lewis et al. (1997) demonstrated how the rhythmic characteristics of the *period* mutants of *Drosophila* could be accounted for by small variations in the components of their postulated feedback loops. Later, Warman (Warman and Lewis, 2001), studying the blow fly *Lucilia cuprina*, successfully translated the rapidly increasing molecular information into more complex feedback loops and components. In this way the dynamic modelling of the clock has kept pace with the expanding catalogue of clock genes and their proteins, and may breath life into the rather static models put forward by the molecular biologists. By this method the integration of quantitative models with molecular information may be fulfilled.

ANNOTATED SUMMARY

1. A dominant goal of chronobiology is to understand the nature of the mechanisms of circadian clocks.
2. The biochemical oscillator hypothesis was a major step in the understanding of circadian timing systems and was the basis of many of the subsequent models. In this simple model the circadian mechanism was seen as a build up and loss of an activity controlling protein on roughly 24 hour timescale.
3. Models should account for the known behaviour of circadian clocks, and should be testable through prediction of new behaviour under experimental conditions.
4. With the increasing molecular knowledge of circadian clocks, it is suggested that quantitative models should reflect the molecular components of the circadian system.
5. A variety of single oscillator models is reviewed, with the emphasis on control systems representations in which circadian oscillations are generated by time-delayed negative feedback systems.
6. The single oscillator feedback model developed to account for the circadian behaviour of the weta *Hemideina thoracica* mimics many of the basic properties of its endogenous

clock, including free-running behaviour, the responses to dim and bright light, light entrainment and temperature compensation.

7. Single oscillator models do not readily explain the lability of rhythms often exhibited in constant conditions, including spontaneous changes in period and desynchrony, and after-effects of a variety of pre-treatments.
8. These more complex patterns of rhythmic behaviour are best explained by models in which clocks are seen to be populations of loosely coupled oscillators. Each unit oscillator has its own periodicity, and in combination with the others creates rhythmic output. The population may however spontaneously desynchronise in constant conditions or be forced apart by critically timed perturbations.
9. The final development of the modelling process sees insect circadian clocks as systems of interacting populations of oscillators, often referred to as X-Y systems.
10. In the *Hemideina* model, the Y system controls the timing of locomotor activity and the X system regulates the cuticle growth rhythm. The two systems are weakly coupled, and in constant conditions may interact to produce day-skipping, scalloping and spontaneous period increase.
11. The achievement of the goals of chronobiology will come about through the integration of molecular and quantitative modelling.

This Page Intentionally Left Blank

CHAPTER 8

CIRCADIAN RHYTHMS: PHOTORECEPTOR AND CLOCK LOCATION

Why has not man a microscopic eye? For this plain reason, man is not a fly.
Alexander Pope

CONTENTS

Introduction	245
A. <i>Clocks Controlling Overt Behavioural Rhythms</i>	246
1. Cockroaches	247
2. Crickets and other Orthoptera	253
3. Beetles	256
4. Flies	256
5. Moths and other orders	259
B. <i>Neural Architecture of Behavioural Clock Systems</i>	260
C. <i>Clocks Controlling Developmental or 'Population' Rhythms</i>	262
Annotated Summary	268

INTRODUCTION

ALTHOUGH individual insect cells may contain or constitute a circadian clock (see Chapter 6), particular *groups of cells* making up tissues or organs may have a particular clock function. The search for the anatomical locations of such pacemakers, and for the photoreceptors that mediate their entrainment to the light cycle, has occupied a central and important position in the study of insect clocks. In this Chapter we will address these problems with reference to overt behavioural rhythms (e.g. general locomotion; Chapter 2) and developmental or 'population' rhythms (e.g. pupal eclosion or moulting; Chapter 3).

In reviewing this work it is useful to refer to the established "**input→ oscillator→ output**" model which, although grossly and almost fatally over-simplified, continues to provide a valid framework. In this simple model, it is supposed that the circadian pacemaker (the 'oscillator') receives an entraining input from the environment, principally light perceived by photoreceptors. The pacemaker itself contains cellular oscillators presenting the canonical circadian properties of persistence, near-24 hour period, temperature compensation etc., and the output pathway connects the clock to the overt rhythm, perhaps *via* slave systems. The validity of this simple model will be discussed later (Chapter 16) but, for now, will offer an adequate framework.

In particular, it suggests that isolation or removal of the relevant photoreceptor(s) would interrupt the input pathway so that the rhythmic function, locomotion or eclosion, would free-run, even in a light-dark cycle, as if the organism were in total darkness. Removal of the pacemaker tissue (the oscillator), on the other hand, would render the animal arrhythmic. Furthermore, if the putative pacemaker were re-implanted into a 'clock-less' and therefore arrhythmic recipient, the overt rhythm would be restored with the period and phase characteristic of the donor. The excised pacemaker may also, in some cases, be capable of supporting circadian rhythmicity, perhaps of a neural output, when maintained *in vitro*. These experiments have all been conducted successfully with insect material.

A. CLOCKS CONTROLLING OVERT BEHAVIOURAL RHYTHMS

Early work in this area was conducted using cockroaches and crickets, presumably because their large size and ease of handling made them suitable experimental subjects. However – in hindsight – some of this work has been invalidated because it was carried out without reference to the predictions arising from the simple 'input-clock-output' model outlined above. This early work, however, will be described below to provide an historical account of the field's development.

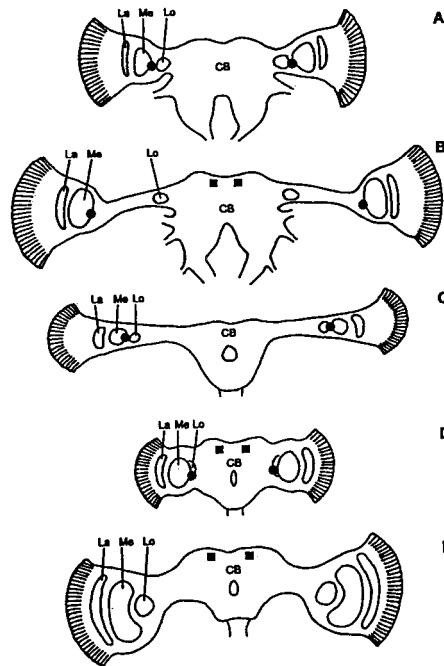


Fig. 8.1. Schematic drawings of the brains of cockroach (A), cricket (B), beetle (C), fly (D) and moth (E) illustrating the optic lobe neuropils (lamina La, medulla Me and lobula Lo) that process and transfer visual signals from the compound eyes to the central brain (CB). The locations of the putative circadian pacemakers are indicated by filled circles in the optic lobes and by squares in the central brain. The central brain pacemaker region comprises neurons in the *pars intercerebralis* or the *pars lateralis*, the neuroendocrine centres of the insect brain. (From Helfrich-Förster et al., 1998).

Enormous advances have been made during the past two decades in uncovering the anatomical locations of the circadian pacemakers controlling locomotor rhythms and their associated photoreceptors, not only in cockroaches and crickets, but in beetles, flies and moths. This material is covered in an excellent recent review by Helfrich-Förster et al. (1998), and may be summarised in Fig. 8.1. Very briefly, the compound eyes are the principal or only photoreceptors in cockroaches, and the circadian pacemakers (bilaterally duplicated) are located in the optic lobes (accessory medulla). In crickets and some other orthopteroid insects, there is evidence, in addition, for extra-optic photoreception and, in some species, for circadian pacemakers outside the optic lobes. Results from flies and moths also indicate extra-optic photoreception and an increasing importance of the central brain as the site of the relevant pacemaker(s). A more detailed account of circadian pacemaker location, and of the input and output pathways in flies (particularly *Drosophila melanogaster*), are to be found in Chapter 4. The increased importance of the central brain in Holometabola may have evolved as a consequence of 'complete' metamorphosis (Truman, 1971b).

1. Cockroaches

Photoreceptors. The rhythm of locomotor activity in cockroaches free-runs in the absence of environmental *Zeitgeber* (see Chapter 2) but becomes entrained to a 24-hour cycle of light and darkness so that most of the activity occurs during the dark phase. Candidate photoreceptors for such entrainment are the compound eyes, the ocelli, or an extra-optic photosensitivity, perhaps in the brain itself. In the search for the photoreceptors in cockroaches attention has been directed at all three.

Working with *Periplaneta americana*, Cloudsley-Thompson (1953) claimed that painting over eyes and ocelli caused the insects to become arrhythmic. Harker (1955, 1956) extended these observations and directed particular attention to the ocelli. She claimed that destroying or covering the ocelli, or cutting the ocellar nerves, resulted in a 'gradual loss of the normal rhythm'. After a period in DD cockroaches with cut ocellar nerves would not 'take up' a new rhythm under new light conditions. According to Harker, covering the compound eyes but leaving the ocelli intact had no effect. These results seemed to implicate the ocelli as the principal if not the only photoreceptors, and Harker (1956) claimed that they were 'directly connected with the establishment of the rhythm by the external factors of light and darkness'. On the basis of subsequent work (see below), however, one would expect that elimination of the photoreceptor would cause the rhythm to free-run in LD, not the appearance of arrhythmia. The results were therefore equivocal.

Roberts (1965a) painted over the compound eyes and ocelli of *Leucophaea maderae* and *Periplaneta americana* with a mixture of lacquer, carbon black and beeswax, and came to quite an opposite conclusion. The insects were also maintained in running wheels, an apparatus that provided longer and more clear-cut activity records than the rocking actographs used by Harker. Figure 8.2 shows the record of a specimen of *L. maderae* initially entrained to LD 12:12. On day 20 the compound eyes were painted over but the ocelli left intact: the insect free-ran, even though still in a light-cycle, clearly demonstrating that the photoreceptors had been eliminated. On day 50 the paint was removed and the rhythm re-entrained to LD 12:12. Subsequent removal of the ocelli on day 68 had no effect on entrainment. These results clearly indicated that the photoreceptors for entrainment were the compound eyes and not the ocelli, but did not rule out the possibility of direct photostimulation of the brain.

These observations were later confirmed and extended by Nishiitsutsuji-Uwo and Pittendrigh (1968a). They found that surgical removal of the ocelli had no effect on

entrainment, but that painting over the entire head, or bilateral section of the optic nerves, caused the insects to free-run in LD 12:12. In order to investigate the question of *direct* photostimulation of the brain, insects with their entire head capsule painted had the paint removed from the translucent antennal sockets, or had a glass 'window' inserted into the vertex of the head, above the protocerebrum. This procedure caused entrainment to be re-established. It was concluded, however, that light was probably not absorbed directly by the brain. For example, in animals with a 'window' and their optic nerves cut, entrainment failed - suggesting that light entering the glass window scattered within the head capsule and activated the ommatidial elements.

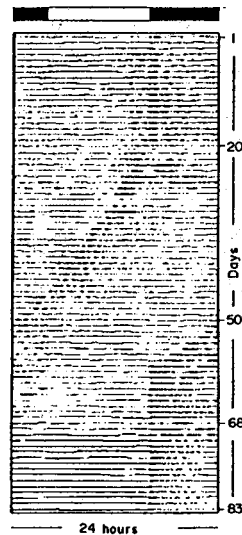


Fig. 8.2. Record of a rhythm of locomotor activity of a single cockroach, *Leucophaea maderae*, maintained in a light/dark cycle (LD 12:12) for 83 days. On day 20 the compound eyes were painted with black lacquer; on day 50 the paint was peeled off; and on day 68 the ocelli were surgically removed. The position of the light and dark fractions of the LD regime is indicated at the top of the figure. (From Roberts, 1965a.) (Copyright 1965 by the American Association for the Advancement of Science.)

Ball (1971) confirmed that the compound eyes and not the ocelli were the principal photoreceptors in *P. americana*. Since earlier studies had also suggested that the terminal abdominal ganglion was light sensitive (Ball, 1965), the role of this organ in the control of rhythmicity was investigated. When the terminal ganglion was occluded more activity was found to occur in the light phase, but transection of the ventral nerve cord did not alter the locomotor rhythm. In subsequent experiments with *Blaberus craniifer* maintained in running wheels at LD 12:12, Ball (1972) inserted a small glass window over the protocerebrum and then covered the rest of the head capsule with an opaque black wax. In twelve out of fourteen insects so treated, entrainment to the light-cycle was maintained for 3 to 6 weeks. In the remaining two cases arrhythmia occurred. In view of Nishiitsutsuji-Uwo and Pittendrigh's (1968a) observations with *L. maderae*, however, the result remains equivocal: light entering the window could have activated the ommatidia directly.

Although the dominant role of the compound eyes in the entrainment of cockroach activity rhythms seems certain, Rivault (1976) claimed that both eyes *and* ocelli were involved

in the *initiation* of rhythmicity. Males of *P. americana*, hatched and reared in constant light, were transferred to LD 12:12. In some insects the eyes, ocelli, or periantennal areas were occluded with black paint, in others the ocelli, the eyes, or both ocelli and eyes, were destroyed by cautery. The results were rather equivocal: only a small minority of insects with ocelli or eyes painted developed rhythms in LD, whereas all of those with cauterised eyes and ocelli became rhythmic. It was concluded that both types of photoreceptor may be important in the initiation of rhythmic activity, with light leaking through the clearer periantennal cuticle also playing a part in light transmission.

In summary, however, the most reliable evidence suggests that the compound eyes are the principal, if not the only, photoreceptors involved in cockroaches.

Pacemaker location. In 1954 Harker reported some results of parabiosis experiments with *P. americana* in which an upper cockroach, previously maintained in LD 12:12 and therefore rhythmic, but with its legs removed, was joined by its pronotum to a lower insect which was intact but rendered 'arrhythmic' by spending its entire life in LL. She found that the motile insect showed a circadian rhythm of activity corresponding to that of the top insect and concluded that "a secretion, carried either in the blood or tissues, is involved in the production of a diurnal rhythm of activity in the cockroach". In subsequent papers Harker (1955, 1956) identified the sub-oesophageal ganglion as the source of the proposed hormone. Using a headless and therefore arrhythmic insect as a 'test-bed' she implanted a sub-oesophageal ganglion from a rhythmic donor into the abdomen just lateral to the heart. When the recipient was then kept in any light condition (LL, DD or reversed LD) it showed a 'normal rhythm' with its active phases corresponding to those of the donor. The source of the secretion was later traced to two pairs of neurosecretory cells, one on either side of the sub-oesophageal ganglion. It was concluded that the sub-oesophageal ganglion could carry on secreting rhythmically after all nervous connections had been broken; it was therefore an autonomous endocrine 'clock'. It was also found that surgical removal of the *corpora allata* (allatectomy) caused a gradual loss of the rhythm because the operation broke two small nerves which conducted neurosecretory material from the *corpus cardiacum* to the sub-oesophageal ganglion (Harker, 1960 a, b). This supply of material was considered essential for the continued running of the clock. At the time these observations were made they constituted one of the most important findings in the study of insect clocks because they were the first to claim an anatomical locus for a circadian pacemaker. However, they have generally failed to correspond with the findings of subsequent investigators.

Roberts (1965b, 1966) criticised Harker's experiments on a number of grounds. (1) Her parabiosis experiments were conducted without controls in which the pairs of cockroaches lacked haemocoel connection, so that apparent 'transfer' of the rhythm could have been due to mechanical stimulation. (2) The fact that he (Roberts, 1960) had demonstrated the persistence of rhythmic locomotor activity in constant light of quite high intensity, implying that Harker's lower animals were not, in fact, arrhythmic. (3) That Harker had not paid sufficient attention to the respective phases of the donor and recipient. He pointed out that experiments involving the transfer of sub-oesophageal ganglia, or any other organ, should be conducted between insects purposely entrained to light-cycles out-of-phase with each other. In the absence of this precaution the evidence merely suggested that the sub-oesophageal ganglion was necessary for the 'expression' of the rhythm. Similar criticisms were later voiced by Nishiitsutsuji-Uwo et al. (1967) and Cymborowski and Brady (1972).

In his own experiments, Roberts (1966) failed to reinstate the rhythm in decapitated *P. americana* by implanting sub-oesophageal ganglia from rhythmic donors (twenty attempts). He

also found that allatectomy or even removal of the entire retrocerebral complex failed to interfere with the rhythm, and that the rhythm could not be reinstated with sub-oesophageal ganglion transplants to decapitated insects which were then subjected to a 24-hour sinusoidal temperature cycle (19° to 27°C). He therefore failed to confirm Harker's observations concerning the role of the sub-oesophageal ganglion. He did, however, evoke arrhythmicity in both *Leucophaea maderae* and *Periplaneta americana* by surgical bisection of the pars intercerebralis thus focusing attention on the brain itself.

Brady (1967a, b) carried out a careful study designed to reconcile some of the conflict between the results of Harker and Roberts. This investigation involved larger numbers of insects, microcautery of neurosecretory cells, subsequent autopsy by histological examination, and careful attention to phase differences between donors and recipients. He found that complete removal of the corpora cardiaca (as revealed by autopsy) was almost impossible; this operation broke the nervous connection from the corpus cardiacum to the sub-oesophageal ganglion. Nonetheless, the rhythm persisted, thereby confirming Roberts' (1966) contentions. Destruction of the median neurosecretory cells of the pars intercerebralis of thirty-seven insects by microcautery left seven arrhythmic, twelve possibly rhythmic and sixteen clearly rhythmic; the remaining two died. Autopsies revealed that cockroaches were able to maintain a rhythm after massive if not total reduction of their neurosecretory cells. In his experiments involving sub-oesophageal ganglion transplants, Brady (1967b) maintained donors and recipients both in LD 12:12 before the operation, but with the light to dark transitions for recipients 5 to 10 hours later than that for donors. Sub-oesophageal ganglia were implanted into forty-eight headless recipients that were then maintained in DD. Of the twenty-nine 'acceptable' records, the majority were arrhythmic. Only in two cases was the greatest part of their recorded activity at nearly the same time each day as the donor's previous activity peak. Cautery of the neurosecretory cells in sub-oesophageal ganglia failed to make the cockroaches arrhythmic. Brady's data therefore also failed to confirm Harker's claim.

Nishiitsutsuji-Uwo et al. (1967) also used larger numbers of insects and checked the results of their various surgical procedures by postmortem histological examination. Surgical removal of the pars intercerebralis in forty-seven insects caused arrhythmicity in nineteen cases and some rhythmicity in the remaining twenty-eight. Subsequent autopsy of three of the nineteen arrhythmic cockroaches revealed that no neurosecretory cells remained, whereas eleven of the twenty-eight partially rhythmic animals so examined were found to contain at least some of these cells. It was concluded, therefore, that ablation of the pars intercerebralis caused arrhythmicity provided that all of these cells were removed. This result - which is in conflict with that of Brady (1967b) - was interpreted as evidence for a relationship between the neurosecretory cells and the circadian rhythm, and therefore for a hormonal link.

In a subsequent paper Nishiitsutsuji-Uwo and Pittendrigh (1968b) produced the best evidence that the driving oscillation is located in the brain. More specifically, they found that cutting the optic nerves (of *L. maderae*) caused the rhythm to free-run in LD 12:12, but cutting the optic tracts (between the optic lobes and the rest of the brain) resulted in arrhythmicity. They concluded, therefore, that the 'clock' was located in the optic lobes, and that these structures required connection to the compound eyes for entrainment and connection to the rest of the protocerebrum for the mediation of the locomotory rhythm. Since they also found that destruction of the neurosecretory cells of the pars intercerebralis resulted in arrhythmicity, they favoured an output from the clock to the locomotory centres that included a humoral link.

In a review attempting to collate all published observations, Brady (1969) concluded, however, that a neural channel between the optic lobes and the thoracic centres was involved. He pointed out that there were only three routes by which such control could reach the thoracic

ganglia and hence the legs. These are (1) by hormones in the blood, (2) by hormones carried in nerve axons and (3) by normal electrical impulses in the nerves. The fact that virtually all of the cephalic endocrine tissue could be removed without stopping the rhythm makes the first seem unlikely. The crucial experiments to demonstrate nervous control would therefore seem to be cutting the ventral nerve cord at various points along the ganglionic chain. These operations are shown in Fig. 8.3. Cutting the ventral nerve cord behind the thorax (cut C) was found to have no effect on the activity rhythm, as might be expected (Nishiitsutsuji-Uwo and Pittendrigh, 1968b). Cutting the connectives between the thoracic ganglia (cut D) had a greater effect when performed between the pro- and the mesothoracic ganglia than between the meso- and metathoracic ganglia (Nishiitsutsuji-Uwo and Pittendrigh, 1968b). Cutting the nerve cord between the sub-oesophageal ganglion and the thorax (cut E) caused complete loss of the rhythm (Brady, 1967b; Nishiitsutsuji-Uwo and Pittendrigh, 1968b). The results of cuts A and B, between compound eyes and optic lobes and between optic lobes and brain, respectively, have already been noted. Unfortunately the really crucial test of cutting the circum-oesophageal connectives (cut F) proved equivocal. If the operation was performed with *P. americana*, activity became so intense that it became difficult to interpret the records (Brady, 1967b); with *L. maderae*, however, the same operation clearly caused arrhythmicity (Roberts et al., 1971). These results all constitute strong evidence that the driving oscillation located in the optic lobes has a nervous (i.e. electrical) rather than a hormonal output.

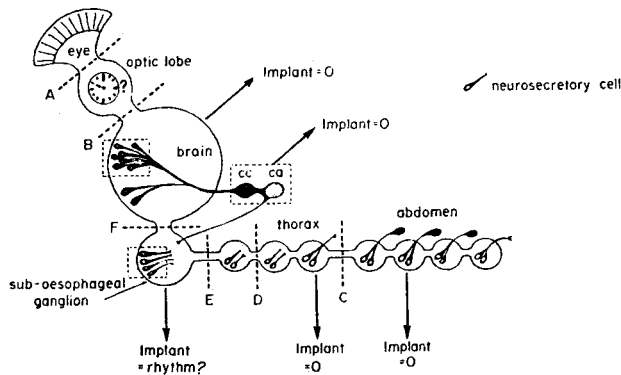


Fig. 8.3. Synopsis of early experiments on the control of the circadian locomotor activity rhythm in cockroaches. The ganglia of the central nervous system are represented by linked spheres. Neuroendocrine tissue, including known neurohaemal organs, are indicated in black. Dotted boxes represent endocrine tissue that can be removed without altering the rhythm. Arrows show organs transplanted from rhythmic donors to headless arrhythmic recipients: 0 signifies that the host shows no detectable rhythm. Heavy broken lines are cuts made in the nerve trunks: cuts B, E, F, or splitting the protocerebral lobes bilaterally, apparently stop the rhythm; cuts A, D, C, or splitting the pars intercerebralis mid-sagittally, do not. cc, *corpora cardiaca*; ca, *corpora allata*. (Redrawn from Brady, 1969.)

Roberts (1974) attempted to locate the driving oscillation in *L. maderae* and *P. americana* to a more precise site within the optic lobes. Surgical removal or destruction of different synaptic areas suggested that the two innermost elements (the lobula and the medulla) were crucial in the control of rhythmicity. Removal of the outer synaptic area (the lamina), on the other hand, caused the animals to remain rhythmic but to free-run in LD; for this reason the lamina was considered essential in the coupling between the photoreceptors (the compound eyes) and the clock.

By the late 1970s observations by Sokolove (1975), Page et al. (1977) and Page (1978) had confirmed the importance of the optic lobes in maintaining rhythmicity, and showed that the bilaterally paired pacemakers in the optic lobes were mutually coupled *via* the pars intercerebralis of the central brain. Roth and Sokolove (1975) found histological evidence for direct monosynaptic connections between the two optic lobes of *L. maderae*. Using electrolytic lesions of various parts of the optic lobe, Page (1978) located the pacemaker with a greater precision. After the removal of one optic lobe, the other lobe was lesioned either in the top third, or in the bottom two-thirds. Only when lesioned in the lower part were cockroaches rendered arrhythmic, and the pacemaker was thought to lie close to the lobula. It was concluded that the bilaterally paired pacemakers were coupled and mutually accelerated *via* a polysynaptic pathway across the mid-brain, and that entrainment was by its contralateral eye. More recently, Homberg et al. (1991) located the pacemaker to a small neuropil between the medulla and lobula, called the *accessory medulla* (Fig. 8.1) (see Section B).

Although the optic lobes of cockroaches contain the most important circadian pacemakers for locomotor rhythmicity, Page (1985) showed that a pacemaker(s) outside the optic lobes was also involved. Bilaterally lobectomised cockroaches became arrhythmic in constant conditions, but a rhythm could be re-imposed by a temperature cycle of only 6°C in amplitude. Since the phase relationship (ψ) between locomotor activity and the temperature cycle was dependent on both the relative durations of the warm and cold phases, and on cycle length (T), 'true' entrainment was indicated. A damped circadian oscillator outside the optic lobes was thought to be involved. Evidence for such oscillators are also evident in crickets (see below).

Optic lobe transplants. In a very elegant experiment, Page (1982) raised cockroaches in two different light cycles, LD 11:11 (T = 22 hours) or LD 13:13 (T = 26 hours), treatments that resulted in two groups of insects, one with a short period (mean τ = 22.7 hours) and the other with a long period (mean τ = 24.2 hours). These differences were maintained for at least 5 months in DD and were considered 'permanent' (see Chapter 2, section C). Surgical removal of the optic lobes – which resulted in behavioural arrhythmicity – was then followed by a reciprocal transplantation of optic lobes from short-period to long-period insects, and *vice versa*. Four to 8 weeks later rhythmicity was restored, post-mortem histological examination revealing that the transplanted optic lobes had regenerated appropriate connections to the brain. Since the period and phase of the recipient's adopted rhythm were close to that of the donor in each case, this experiment provided unequivocal evidence that the optic lobes contained the circadian pacemakers.

In a subsequent paper (Page 1983a) it was shown that bilateral section of the optic tracts (initially causing arrhythmic behaviour) led to resumed rhythmicity after connections to the brain had regenerated, 3 to 5 weeks later. Confirmation that resumption of the rhythm was due to this regeneration was obtained by (1) histological examination, (2) insertion of a glass barrier between optic lobes and brain to prevent neural connections, and (3) extracellular recordings which showed recovery of light-evoked activity in the cervical connectives. An entrainable circadian pacemaker was thus shown to reside in each optic lobe after its separation from the brain. Page (1983b) later showed that an optic lobe pacemaker, isolated by optic tract section, was capable of regenerating connections across the brain to its contralateral partner.

Neural activity from isolated pacemakers. Final proof that the optic lobes of cockroaches contained a circadian pacemaker was provided by Colwell and Page (1990) who recorded a robust rhythm of spontaneous multi-unit neural activity from isolated optic lobes of *L. maderae* maintained *in vitro* (culture dishes at 23°C, continuous light). Wells et al. (1985) had earlier

recorded rhythms in electroretinogram (ERG) amplitude of *L. maderae*, which persisted after isolation of the optic lobes from the mid brain, by cuts made proximal to the distal edge of the lobula. Since bisection of the optic lobe *distal* to the lobula abolished the rhythm, it was concluded that the circadian pacemaker controlling the ERG rhythm was, like that controlling locomotor rhythmicity, close to the lobula region of the optic lobe.

2. Crickets and other Orthoptera

As in cockroaches, the most important photoreceptors for entrainment in crickets (locomotor and singing rhythms) are the compound eyes, and the main pacemaker centres are in the optic lobes. In crickets, however, there is also persuasive evidence for extra-optic photoreception and for additional pacemaker sites, perhaps in the central brain.

Compound eyes as photoreceptors. In the cricket *Teleogryllus commodus*, the compound eyes appear to be the most important, if not the only, photoreceptors involved in entrainment (Loher, 1972; Sokolove and Loher, 1975). Males of this species possessed a well-defined stridulatory rhythm with singing commencing about 2 hours after dark. In DD the rhythm free-ran with a natural period (τ) of 23 hours 36 minutes; in LL τ was 25 hours 40 minutes. Surgical removal of the compound eyes or severance of the ommatidial nerves caused the insects to free-run in LD or in LL with a period similar to that occurring in unoperated animals maintained in DD; removal of the ocelli, on the other hand, was considered inconsequential. The compound eyes also play a dominant role in *Gryllus bimaculatus* (Tomioka et al., 1990; Tomioka and Yukizane, 1997) and in the New Zealand weta, *Hemideina thoracica* (Waddell et al., 1990). These results are directly comparable to those for the cockroach *Leucophaea maderae* obtained by Roberts (1965) and Nishiitsutsuji-Uwo and Pittendrigh (1968a).

In more recent studies with *Gryllus bimaculatus*, the role of the compound eye has been investigated by the technique of partial reduction of the number of ommatidia. Using crickets in which one eye was rendered inoperative by severance of its optic stalk, the other eye was reduced in size by removing either the dorsal or the ventral portion, or both dorsal and ventral portions, leaving a central band intact. Stable entrainment occurred in all groups, suggesting that all areas of the eye were equivalent. After a 6-hour phase advance in the entraining light cycle, the number of non-steady state transients (see Chapters 2 and 3) was inversely correlated with the number of ommatidia remaining. However, entrainment could still occur when only 262 ommatidia remained from a total of over 3500 in the intact eye. In a later paper, Tomioka and Yukizane (1997) showed that the dorso-caudal area of the eye was a specific region receiving photic signals that were transmitted to the contralateral pacemaker.

Extra-optic photoreception. Although the compound eye is the most important photoreceptor for circadian entrainment in crickets, there have been several claims, and some evidence, for extra-optic photoreception. For example, working with the house cricket *Acheta domesticus*, Nowosielski and Patton (1963) claimed that entrainment to a 'new' light-cycle could only be prevented by blacking out both compound eyes *and* ocelli. Out of twenty-seven insects with their compound eyes occluded, thirteen showed no distinct re-entrainment, nine of these becoming arrhythmic. The authors concluded that both types of photoreceptor were necessary although eyes played the dominant role.

In the grasshopper *Chorthippus curtipennis*, Loher and Chandrashekar (1970) suggested that photic entrainment of the oviposition rhythm continued when both ocelli and compound eyes were destroyed or covered with an opaque wax, or even when the entire head capsule was

covered with a thick coating of the waxy material. They concluded that an *extra-cephalic* photoreceptor was in operation. Similarly, Godden (1973) found that ablation of the compound eyes in the stick insect *Carausius morosus* failed to interfere with entrainment to LD 12:12 and suspected an extra-optic receptor. An extra-optic photoreceptor was also suggested for entrainment of the stridulatory rhythm in species of *Ephippiger* (Dumortier, 1972). In these insects, covering the entire head with paint or an aluminium 'cowl', or surgical destruction of the compound eyes and ocelli by electrocoagulation, failed to prevent entrainment to a light-dark cycle. Blinded individuals also re-entrained to a reversed LD cycle. The exact location of the photoreceptor in *Ephippiger* was not clear, but it may be in the brain: it is certainly head-located because illumination of the head with a light-dark cycle will entrain, whereas similar illumination of the body will not. However, the rather high intensity light used in these experiments might have led to light scattering within the head capsule and direct illumination of the ommatidia.

The possible role of the ocelli was further examined in *Teleogryllus commodus* by Rence et al. (1988). Using low intensity continuous illumination (LL), the insects were shown to display a free-running rhythm of singing activity with $\tau \sim 24$ hours. Severance of all three ocellar nerves significantly slowed τ suggesting reduced perception of the available light. Comparisons of electroretinograms (ERGs) of crickets with functional ocelli, with occluded ocelli, and with ocelli occluded but subsequently uncovered, indicated that these 'simple' eyes might *modulate* perception of light. They may therefore play an *indirect* role in circadian photoreception, augmenting the sensitivity of the primary photoreceptors, the compound eyes.

Working with the band-legged ground cricket, *Dianemobius nigrofasciatus*, Shiga et al. (1999) found that removal of the compound eyes allowed some crickets to entrain to a light cycle (LD 13:13 or LD 12:12). Entrainment still occurred, moreover, in a few crickets which had both their eyes *and* ocelli removed; this suggested possible extra-retinal circadian photoreception.

In summary, therefore, evidence exists for extra-optic photoreception in crickets, but the possible role of the ocelli remains equivocal.

Pacemaker location. In the cricket *Teleogryllus commodus*, severance of both optic lobes caused a breakdown in the stridulatory rhythm, suggesting that the clock(s) were located within these structures (Loher, 1972; Sokolove and Loher, 1975). Section of one optic tract, however, did not affect the rhythm. In this species, therefore, both the location of the photoreceptors in the compound eyes, and the location of the pacemakers within the optic lobes, makes it directly comparable to the cockroaches discussed earlier. An optic lobe pacemaker was also indicated in *Gryllus bimaculatus* (Tomioka and Chiba, 1986; Chiba and Tomioka, 1987), the weta *Hemideina thoracica* (Waddell et al., 1990) and the ground cricket *Dianemobius nigrofasciatus* (Shiga et al., 1999).

In *Gryllus bimaculatus* the two outermost neuropils of the optic lobe (the lamina and medulla) are connected to the innermost area, the lobula, by a long 'stalk'. Surgical removal of the lamina-medulla complex, or bilateral section of the stalk, rendered insects arrhythmic in LD 12:12 for both locomotor and singing behaviour (Tomioka and Chiba, 1984; Tomioka, 1985). Recording efferent neural activities from the isolated lamina-medulla complex using a suction electrode attached to the stalk, showed the importance of this complex as a pacemaker centre (Tomioka and Chiba, 1986). In this study, multiple unit activities from the stalk showed a clear circadian rhythm in LL with an endogenous period greater than 24 hours. Removal of the cerebral lobes or the sub-oesophageal ganglion did not affect this rhythm. Therefore, the

lamina-medulla region of the optic lobe was shown to contain an important neural pacemaker generating a rhythm without input from other parts of the CNS.

In some crickets there is persuasive evidence for circadian pacemaker(s) *outside* the optic lobes. For example, in *Teleogryllus commodus*, Rence and Loher (1975) showed that although bilateral lobectomy led to arrhythmic singing behaviour, exposure of these arrhythmic insects to a daily temperature cycle (12 hours at 25° followed by 12 hours at 35°C) in constant light, re-introduced a rhythm of singing that subsequently free-ran. 'True' entrainment was indicated by the occurrence of transients before the adoption of a new steady state, and the inability of such crickets to synchronise their singing rhythms to cycles as long as 30 hours, which were considered to be outside the primary range of entrainment (see Chapter 2). These results indicated the presence of an additional pacemaker – perhaps a damped oscillator – outside the optic lobes and possibly in the central brain.

Comparable data suggesting circadian pacemakers outside the optic lobes are also available in other crickets. In *Gryllus bimaculatus*, Tomioka (1985) showed that rhythmicity persisted in some individuals after bilateral ablation of the lamina and medulla, or after transection of both optic stalks. In a later paper, Tomioka and Chiba (1989) removed lamina-medulla complexes from 7th instar nymphs and then recorded their locomotor activity after the imaginal moult. Despite lacking the crucial elements of the optic lobes, about half of these insects displayed a rhythm with the onset of activity phase-leading the light, therefore indicating true entrainment. In DD, about 21 per cent of such crickets free-ran with a circadian period, a further 26 per cent showed ultradian rhythms (τ about 4 to 12 hours), whilst the remainder were arrhythmic.

Cymborowski (1981) produced some of the oldest evidence for the existence of a pacemaker outside the optic lobes. In the house cricket, *Acheta domesticus*, he found that radio-frequency destruction of neurosecretory cells (NSC) in the pars intercerebralis of the brain resulted in arrhythmic behaviour. Transplanting brains from naturally occurring short- or long-period crickets to the abdomens of NSC-ablated recipients caused a proportion of the latter to resume rhythmic behaviour with a period characteristic of the donor. Implantation of brains with their NSCs destroyed did not induce a rhythm in the host. Since adoption of the rhythm was virtually instantaneous, and post-mortem histological examination revealed no new neural connections, a hormonal output from a brain-centred clock was suspected. How this proposed hormonal clock in the brain relates to a possible neural clock in the optic lobes remains unknown.

Information on the anatomical location of the driving oscillation in other orthopteroids is comparatively sparse. Optic lobe removal in the ground cricket, *Dianemobius nigro-fasciatus*, led to arrhythmicity in both LD 13:13 and DD (Shiga et al., 1999) whereas a similar operation with the weta, *Hemideina thoracica*, left some individuals capable of entrainment by a temperature cycle (Waddell et al., 1990). Early, inconclusive results with the lubber grasshopper *Romalea microptera* showed that the rhythm of locomotor activity was unaltered by allatectomy. Removal of the sub-oesophageal ganglion caused an apparent loss in rhythmicity, but this was not restored by its subsequent re-implantation (Fingerman et al., 1958). Eidmann (1956) found that the activity rhythm of the stick insect *Carausius morosus* could not be changed by removal of the corpora allata, the corpora cardiaca, or the optic lobes, or by implantation of the brain or sub-oesophageal ganglion. Extirpation of the brain or its protocerebrum, however, abolished the rhythm, as did severance of the circum-oesophageal commissures.

3. Beetles

In beetles, as in cockroaches and crickets, the most important circadian pacemakers are in the optic lobes. In the ground beetle *Carabus problematicus*, for example, bilateral ablation of the optic lobes led to behavioural arrhythmicity (Balkenohl and Weber, 1981).

Circadian rhythms have also been described in long-period electroretinogram (ERG) recordings. Using the desert beetle *Blaps gigas*, Koehler and Fleissner (1978) followed ERG rhythms by using 30 ms 'test' flashes of light every 30 minutes. In continuous darkness (DD), sensitivity to the light flashes was 10 to 100 times greater during the subjective night than during the subjective day. The bilaterally arranged circadian pacemakers controlling the two eye rhythms were only very loosely coupled, and under some circumstances showed internal de-synchronisation (see Chapter 6). Fleissner (1982) also showed that the carabid *Pachymorpha* (= *Anthia*) *sexguttata* displayed an ERG rhythm and a rather weaker rhythm of locomotor activity, both probably controlled by the same (bilaterally arranged) optic lobe pacemakers. Severance of the optic tracts between lobula and brain left the two pacemakers intact, but coupling between them was abolished. Eye rhythms also continued after removal of the entire brain; the rhythm was abolished, however, if the lobula was damaged. The pacemaker was located in the optic lobe, between the lobula and the medulla.

Frisch et al. (1996) used an antibody raised against *Drosophila period* protein (PER) (see Chapter 4) in an attempt to label cells with a possible pacemaker function in *P. sexguttata*. Staining was detected in distinct sets of neurons in the optic lobes and the central brain, and also in some glial cells in the optic lobes. In most cases, neuronal perikarya, axons and terminal regions of the axons were labelled, but PER usually remained stubbornly cytoplasmic. Only in a few small neurons close to the accessory medulla was nuclear staining demonstrated. The pacemaker tissue was thought to lie between the medulla and the lobula, close to the accessory medulla. Immunoreactivity to crustacean pigment dispersing hormone (PDH) was found in some PER-staining cells, but not in all; the significance of these observations will be discussed below (see Section B).

Compound eyes of beetles may act as photoreceptors for circadian entrainment, but Fleissner et al. (1993) described - in the carabid *P. sexguttata* and the tenebrionid *Zophobas morio* - novel non-visual photoreceptors that might also perform this function. They found, deep within the head capsule, close to the dorsal rim of the lobula, and beneath a clear 'window' in the integument, an elongated organ 20 to 40 μm wide and more than 30 μm in length which contained rhabdomere-like structures and which sent fibres to the accessory medulla, the putative optic lobe pacemaker. A second receptor organ displaying the same fine structure was found near the second optic chiasm. These novel photoreceptors, which were distinct from previously known 'internal stemmata', also stained with antibodies raised against bovine rod-opsins and retinal S-antigen (arrestin). Therefore, at least some of the criteria for a circadian photoreceptor were satisfied, and the structures qualify as extra-retinal photoreceptors.

4. Flies

Photoreceptor and pacemaker location has been studied in several groups of Diptera including mosquitoes, fruit flies, house flies and blow flies. Unlike similar studies in cockroaches, crickets and beetles, isolation or removal of the compound eyes (and ocelli) and optic lobes was found to leave persistent circadian rhythms of locomotor activity and their entrainment to the light cycle intact. Although flies may possess multiple photoreceptors and

pacemaker sites, these experiments strongly suggest that 'organised' photoreceptors and optic lobes are *not essential*, focussing attention on the central brain.

Working with the mosquito *Culex pipiens pallens*, Kasai and Chiba (1987) showed that optic lobe ablation left intact circadian rhythms that free-ran in DD with a period τ of 20 to 23 hours. Under continuous light, however, τ became 14 to 15 hours (or perhaps a bimodal rhythm with τ 28 to 30 hours), and entrainment also occurred in a light cycle, indicating the existence of an extra-ocular photoreceptive pathway. Similar results were obtained by Helfrich et al. (1985) in an earlier study on the house fly, *Musca domestica*. Under constant conditions (continuous low intensity red light at 20°C) about half of the flies continued to show circadian rhythms of locomotor activity after bilateral section of the optic tracts or surgical removal of both optic lobes, indicating that the optic lobes were not essential for continued rhythmicity. Of the remaining flies, 30 per cent were apparently arrhythmic, but 20 per cent showed the persistence of several rhythmic components, suggesting a role for the optic lobes in coupling between constituent sub-systems. Since flies deprived of their optic lobes – and hence with compound eyes separated from the brain – also entrained to a light cycle, and showed an apparently 'normal' PRC (to 5 minute light pulses) (see Chapter 2, E), extra-optic photoreception was strongly indicated.

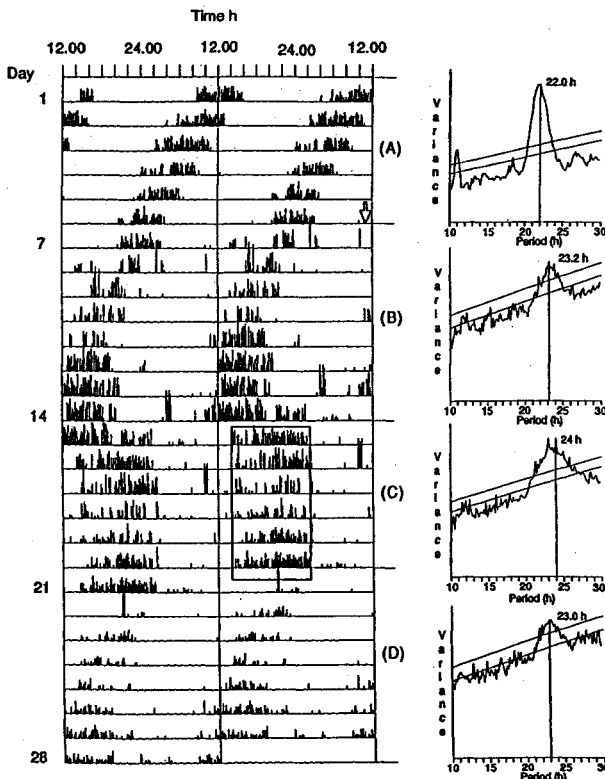


Fig. 8.4. *Calliphora vicina*. Activity record and associated periodogram analyses for an adult female fly whose optic lobes were completely removed during the subjective night (arrow on day 7). Bilobectomy failed to eliminate the free-running rhythm, or entrainment to the light-dark cycle (section C). (From Cymborowski et al., 1994).

In the blow fly *Calliphora vicina*, bilateral optic tract section, or lobectomy, left locomotor rhythms intact and entrainable by light, suggesting pacemaker(s) outside the optic lobes and an extra-optic entrainment pathway (Cymborowski et al., 1994) (Fig. 8.4). Possible photo-receptive inputs to the brain itself were indicated by the demonstration of S-antigen (arrestin) immunoreactivity in various groups of neurons bilaterally distributed in the proto-, deuto- and tritocerebrum (Cymborowski and Korf, 1995). S-antigen involvement in the photic input pathway to circadian pacemakers was also indicated by the injection of S-antigen antibody directly into the blow fly brain, leading to a reduced sensitivity to light (Cymborowski et al., 1996). In LD, some flies failed to entrain and free-ran as though in DD, whereas in LL of quite high intensity some flies free-ran with a period similar to that in DD or in LL of a lower intensity. In this paper particular attention was drawn to four groups of arrestin positive neurons in the lateral section of the brain which might be equivalent to the lateral neurons suspected to be pacemaker cells in *Drosophila melanogaster* (see below, and Section B). On the other hand, Cymborowski and King (1996) demonstrated Fos-like protein in neurons of the *pars intercerebralis* of *C. vicina*. Expression of this protein was only observed when light pulses caused a phase shift in the locomotor rhythm indicating that *c-fos* may play a role in circadian entrainment, as it does in vertebrates. In a later paper, Cymborowski (1998) showed that serotonin (5-hydroxytryptamine, 5-HT) might also be involved in photic transduction. Injection of p-chloro-phenylalanine, an inhibitor of tryptophan hydroxylase, into the haemolymph of blow flies appeared to reduce sensitivity to constant bright light so that flies free-ran as if in DD or dim light. When 5-HT was injected into flies kept in dim LL they became arrhythmic as if in bright LL. These findings suggested a possible role for serotonin as mediator of an input pathway to extraretinal photoreceptors.

Most of what we know about the location of circadian photoreceptors and pacemakers in flies comes from investigations using the fruit fly, *Drosophila melanogaster* (see also Chapter 4). Early studies used various mutations affecting neural structures or sense organs (Mack and Engelmann, 1981; Helfrich and Engelmann, 1983; Helfrich, 1986, 1987). These included: *sine oculis* (*so*), *small optic lobes* (*sol*), *minibrain* (*mbn*), *lobula plateless* (*lop*), *optomotor blind* (*omb*) and the double mutants *mbn;so* and *sol;so*, variously showing reductions to the eyes, optic lobes or brain. For example, in *sine oculis* the compound eyes (and ocelli) are totally lacking in individuals with the greatest gene expression, and in the double mutant *sol;so* the optic lobes are reduced to about 5 per cent of normal. Although these mutations produced profound effects on the flies' morphology, none caused an increase in the number of arrhythmic records over wild type suggesting, initially, that the optic lobes were not the (sole) site of the circadian pacemaker. However, an increased proportion of 'complex' patterns indicated that the optic lobes might be involved in rhythm stability. Since *sine oculis* flies entrained to light, compound eyes (and ocelli) were not essential photoreceptors.

An important contribution has been made by the study of the *disconnected* (*disco*) mutant. In flies carrying this mutation, the compound eyes are frequently separated from the optic lobes. There is also a failure in optic lobe development leading to the degeneration of many neurons, including the so-called *lateral neurons* (LNs), located close to the lateral margin of the brain, and which are the best candidates for pacemaker cells controlling locomotor rhythmicity in adult flies (see section B). Mutations at the *disco* locus resulted in almost total arrhythmicity (in both adult locomotion and pupal eclosion) (Dushay et al., 1989; Hardin et al., 1992). Most of these arrhythmic flies were found to lack the crucial LNs, as revealed by anti-PER staining, indicating a causal relationship between the presence of these cells and overt rhythmicity. Helfrich-Förster (1998) later demonstrated that robust rhythmicity required the

presence of these cells, flies with only one lateral neuron remaining continuing to express a rhythm.

These results appear to indicate that the optic lobes, as in the other flies considered above, are not essential for overt rhythmicity. However, it may be that lateral neurons are equivalent to pacemaker cells in the cockroach accessory medulla, so that they 'belong' to the optic lobe but their somata have moved to a more proximal site within the lateral margin of the brain.

5. Moths and other orders

Working with flight-activity rhythms in males of three species of silk moth (*Antheraea pernyi*, *Hyalophora cecropia* and *Samia cynthia*), Truman (1974) demonstrated three distinct peaks of activity, a brief burst after lights-off, a longer burst in the middle of the night, and a lights-on response. The first two free-ran in DD and were considered endogenous; the third peak was thought to be an exogenous or 'startle' reaction. Extirpation of the compound eyes failed to interfere with the entrainment of the endogenous components, but abolished the exogenous response. Ocelli were not involved. The existence of an extraretinal photoreceptor for entrainment was suggested by covering the entire head with an opaque wax, but leaving the compound eyes exposed; under these conditions the flight rhythm still free-ran in a light/dark cycle.

While the circadian pacemakers governing locomotor activity rhythms in cockroaches and crickets are in the optic lobes (see above), and the output from these lobes to the thoracic centres is neural, the situation in other insects may be different. In silk moths (*A. pernyi*, *H. cecropia* and *S. cynthia*), for example, extirpation of the optic lobes had no effect on the persistence of the flight activity rhythm, but removal of the cerebral lobes led to arrhythmicity (Truman, 1974). In these insects entraining light-cycles are perceived by extra-retinal photoreceptors, the 'clock' is somewhere in the cerebral area, and the output to the thoracic motor centres needs intact neural pathways. Again, neuronal rather than humoral links are involved.

Sauman and Reppert (1996) recently investigated possible pacemaker neurons in *A. pernyi* using immunostaining for *period* protein (PER) (see Chapter 4). Expression of PER was found in just eight cells, four in each brain hemisphere. These were identified as neuro-secretory cells, with PER expression in both cell bodies and in their axons leading to the *corpora allata*. Problems with these cells being 'clock' neurons, however, were apparent. Rather like the situation in beetles (see above) and in *Drosophila* compound eyes, PER remained stubbornly cytoplasmic with no periodic translocation to the nucleus. There was also no time delay between *per* mRNA and the appearance of the protein. However, alternative ways were discussed in which these cells might act as circadian pacemakers; these are outlined in Chapter 4.

Although Truman (1974) concluded that the ocelli were not important as photoreceptors in silkmooths, Eaton et al. (1983) working with flight initiation in the cabbage looper *Trichoplusia ni*, and Wunderer and Kramer (1989) working with mating activity in the arctiid moth *Cretonus transiens*, suggested that the ocelli played a marginal role. In both cases, occlusion of the ocelli caused a delay in the onset of activity. However, as with similar experiments on the role of the ocelli in cockroaches and crickets (see above) the results remain equivocal.

Outside the Lepidoptera, evidence for photoreceptor and pacemaker location is thin. Working with the hemipteran *Graphosoma lineatum*, Nakamura and Hodkova (1998) showed

that bilateral removal of the compound eyes resulted in a loss of behavioural rhythmicity in both LD 18:6 and DD. This may suggest that the compound eye-optic lobe axis is important.

B. NEURAL ARCHITECTURE OF BEHAVIOURAL CLOCK SYSTEMS

Figure 8.5 summarises the organisation of the circadian system timing locomotor and electroretinogram (ERG) rhythms in the cockroach *Leucophaea maderae* as seen by T.L. Page in 1988, and derived from his extensive investigations reviewed in Section A. Each of the two, bilaterally paired optic lobe driving oscillators have three output pathways. One controls the rhythm in ERG amplitude in the eye, a second regulates locomotor activity through a driven system in the midbrain, and a third couples each oscillator to its companion in the contralateral optic lobe. Input pathways are light entrainment from the photoreceptor in the ipsilateral compound eye, and across the brain, from the contralateral optic lobe. This section now describes recent advances in understanding the neural anatomy of such a system, using examples from cockroaches, crickets and flies.

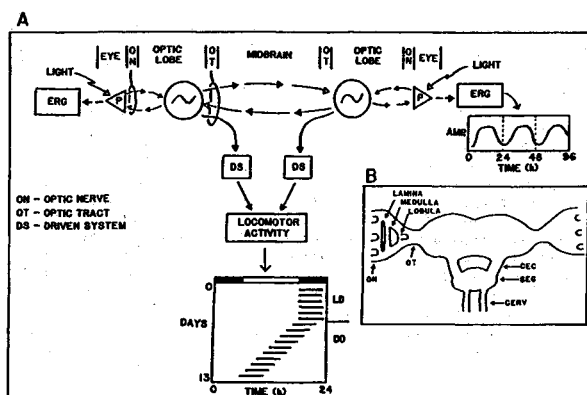


Fig. 8.5. A - Schematic model of the circadian system in the cockroach, *Leucophaea maderae*. There are two, bilaterally paired driving oscillators (pacemakers), each located in one of the optic lobes. Each has three output pathways. One controls a rhythm in ERG amplitude in the eye, a second regulates locomotor activity via a driven system (DS) in the mid-brain, and a third couples the oscillator to its companion oscillator in the contralateral optic lobe. There are two input pathways: a light entrainable pathway from the retina of the ipsilateral eye, and an input from the contralateral optic lobe. B - Schematic drawing of the anterior of the cockroach CNS showing mid-brain, the 3 regions of neuropil of the optic lobe (lamina, medulla and lobula), optic nerve (ON), optic tract (OT), Circumoesophageal connectives (CEC), suboesophageal ganglion (SEG) and the cervical connectives (CERV). (From Page, 1988).

Major advances came with the discovery of the *accessory medulla* and that immuno-cytochemistry using an antiserum raised against the crustacean *pigment-dispersing hormone* (PDH) revealed neurons with extensive axonal arborisations into the medulla, lamina and midbrain that fulfilled many of the criteria qualifying them as pacemaker cells (Homberg et al., 1991). Figure 8.6 shows reconstructions of PDH-immunoreactive neurons in the brains of four insects, a cockroach (*L. maderae*; Stengl and Homberg, 1994), a cricket (*Teleogryllus commodus*; Homberg et al., 1991), and two flies (*Drosophila melanogaster*; Helfrich-Förster, 1997, and *Phormia terraenovae*; Nässel et al. 1991). In all four species PDH-ir processes are

concentrated in the accessory medulla close to the base of the medulla. These cells project to the optic lobes and to the brain. In the optic lobes, fibres undergo extensive arborisation over the medulla and, in *Leucophaea*, *Teleogryllus* and *Phormia*, out to the lamina. PDH neurons also connect to several sites in the central brain, and also across to the contralateral accessory medulla. Details of these networks may be found in the relevant papers.

Using unilaterally lobectomised cockroaches (*L. maderae*), Reischig and Stengl (1997) showed that extirpation of the remaining accessory medulla with its associated PDH-ir neurons caused permanent arrhythmicity. In an experiment reminiscent of that performed earlier by Page (1982), transplantation of the accessory medulla from a donor cockroach to the brain of a host without optic lobes restored locomotor rhythmicity with a period approaching that of the donor. In all such cockroaches, the PDH-ir neurons had regenerated appropriate connections to the protocerebrum. These experiments provided persuasive evidence that the accessory medulla was the site of the circadian pacemaker in cockroaches and crickets, and that regeneration of PDH-ir fibres was necessary for pacemaker function.

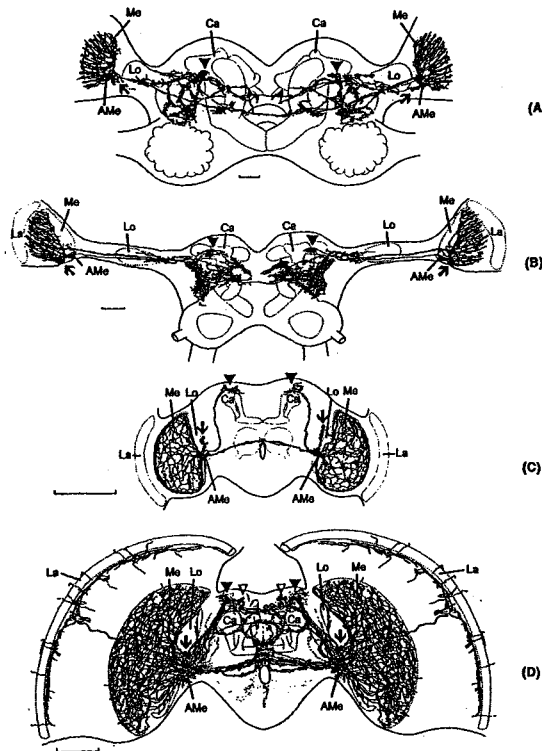


Fig. 8.6. Reconstruction of PDH-ir neurons in the brains of: A – the cockroach *Leucophaea maderae*, B – the cricket *Teleogryllus commodus*, C – *Drosophila melanogaster*, and D – the blow fly *Phormia terraenovae*. Arrows point to the PDH-ir cells located between the medulla (Me) and the lobula (Lo); these cells are concentrated in the accessory medulla (AMe) the presumptive optic lobe pacemaker. In all four species, the PDH-ir cells project distally into the optic lobes with extensive arborisations, and proximally into the central brain. Data are from Stengl and Homberg, 1994 (A), Homberg et al. 1991 (B), Helfrich-Förster, 1997 (C), and Nässel et al., 1991, 1993 (D). (From Helfrich-Förster et al., 1998; see this paper for full details).

In flies, progress was made from two directions. In the blow fly *Phormia terraenovae* and in *D. melanogaster*, Nässel et al. (1991, 1993) described a small number of PDH-ir neurons at the anterior base of the medulla (see Fig. 8.6 C and D). Meanwhile, Siwicki et al. (1988), Ewer et al. (1992) and Frisch et al. (1994), using antibodies raised against the putative 'clock' protein PER (see Chapter 4), demonstrated PER-ir in - among others - groups of neurons called *lateral neurons* whose somata were close to the medulla at the border between the central brain and the optic lobe. These two strands of research were brought together in an important study by Helfrich-Förster (1995) on the co-localisation of PER and PDH in *Drosophila*. This study revealed PER staining in two clusters of lateral neurons - a dorsal group of 3 to 7 cells (the LN_d) and a ventral group of 4 to 10 cells (the LN_v). PER immunoreactivity was demonstrated in the LN_vs together with a fine arborisation over the medulla and fibres to the posterior optic tract and to the superior protocerebrum (Fig. 8.6 C). Double staining for PER and PDH was observed in the ventral lateral neurons (LN_vs). As noted above (section A 4), flies carrying the *disconnected* mutation were found to be rhythmic only when at least one of the LN_vs remained (Helfrich-Förster, 1998) thus attesting to their importance as the circadian pacemaker cells controlling locomotor rhythmicity.

Helfrich-Förster (1997) also investigated PDH-ir neurons in the developing nervous system of *D. melanogaster* using both wild type and *disco* mutants in which LN_vs are usually absent. In wild type, some PDH-ir neurons developed as early as the first instar larva and persisted to the adult, whereas others appeared about halfway through development.

A physiological role for PDH was indicated by Petri and Stengl (1997) who showed that microinjection of PDH into the cockroach medulla, close to the circadian pacemaker, caused phase shifts of the activity rhythm. These phase shifts gave rise to a phase response curve (see Chapter 2) with phase delays ($-\Delta\phi$) of up to 4 hours during the late subjective day and early subjective night, but phase advances ($+\Delta\phi$) up to 2 hours during the late subjective night and early subjective day. This suggested that PDH might act as an input signal or as a component of the clock itself. On the other hand, the fact that PDH immunoreactivity highlighted axons projecting from putative clock cells to presumed target areas in the central brain suggests that PDH may play a role in the clock's output pathway (see Chapter 4). Indeed, early studies by Handler and Konopka (1979) indicated such a humoral output for *D. melanogaster*. In this study, the brains of short-period mutants (*per^S*; $\tau \sim 19$ hours) were transplanted to the abdomens of behaviourally arrhythmic flies (*per^O*). The recipients were then kept in constant conditions and 4 of the 55 survivors found to show a short-period rhythmicity (τ 16 to 20 hours) whilst the remainder, and *per^O* flies receiving *per^O* brains, remained arrhythmic.

Although the compound eyes of *D. melanogaster* are not essential as circadian photoreceptors, it seems that *multiple* photoreceptors are probably involved (Chapter 4; Hofbauer and Buchner, 1989; Helfrich-Förster, 1998). It is possible that the lateral neurons identified as circadian pacemaker cells are themselves photoreceptive. This is also indicated in the blow fly, *Calliphora vicina* (Cymborowski and Korf, 1995), where cells at the border between the optic lobes and central brain, which might be equivalent to the LN_vs in *Drosophila*, show immunoreactivity to arrestin (S-antigen) a component of the phototransduction cascade in both vertebrates and invertebrates.

B. CLOCKS CONTROLLING DEVELOPMENTAL OR 'POPULATION' RHYTHMS

Pupal eclosion. The rhythm of pupal eclosion in *Drosophila* spp. can be initiated and phase-set by light signals at any stage of post-embryonic development (Brett, 1955; Zimmerman and

Ives, 1971). Since the compound eyes and ocelli are only fully differentiated in the pharate adult, and the brain is the only organ that is not extensively reorganised during metamorphosis, it would seem, *a priori*, that the photoreceptors for entrainment - and probably also the clock - lie in the brain.

Attempts to locate the photoreceptors in *Drosophila* pupae have been hampered by the insects' small size. Nevertheless, an early investigation by Zimmerman and Ives (1971) made progress by painting either the anterior or the posterior ends of the puparium of *D. pseudoobscura* with an opaque black paint. Puparia thus treated were then transferred to DD and exposed to dim monochromatic light pulses (15 minutes at 456 nm and 11.33 to 11.49 log quanta $\text{sec}^{-1} \text{cm}^{-2}$) applied 17 hours after the time when dawn would have occurred on day 1. Subsequent examination for the resulting phase-shifts ($\Delta\phi$) (see Chapter 3) showed that light signals falling on the anterior half generated as great a phase shift as light falling on the whole organism, whereas light falling on the posterior half alone had no effect. This result indicated that the photoreceptors for eclosion rhythmicity were located at the anterior end. In earlier experiments using cardboard masks, Kalmus (1938b) reached a similar conclusion.

Although the compound eyes and ocelli become differentiated in the pharate adult they are apparently not required for photoreception. Pittendrigh (unpublished) and Engelmann and Honegger (1966), for example, showed that an eyeless and ocelliess mutant of *D. melanogaster* (*sine oculis*) was able to entrain normally to a light-cycle. Zimmerman and Ives (1971) subsequently repeated this observation using dim monochromatic light. These authors showed that, although the compound eyes of a white-eyed mutant of *D. pseudoobscura* were 100 times more sensitive to light than wild-type because of the lack of screening pigments, no such difference was observed with respect to the sensitivity of the circadian oscillator (see Chapter 3). These observations constituted strong evidence that the 'organised' photoreceptors were not involved: they suggested *extra-optic* light absorption, possibly by the brain.

Is the clock controlling pupal eclosion in *Drosophila* 'the same' as that controlling adult locomotor rhythmicity? In *D. pseudoobscura* there are several differences to suggest different pacemakers (Engelmann and Mack, 1978; see Chapter 6 B) but similar, compelling, data are not available for *D. melanogaster*. In wild type flies both rhythms are regulated by the *period* gene (see Chapter 4), although τ for locomotion is somewhat shorter than that for eclosion. The mutant *ebony*, however, shows arrhythmic patterns of adult locomotor behaviour whilst eclosion is normal (Newby and Jackson, 1991). Conversely, mutations of *lark* are arrhythmic for eclosion but normal for locomotion (Newby and Jackson, 1993). This difference suggests that *lark*, for example, is part of a downstream process, or output, to eclosion. Since rhythmic transcription of *lark* does not persist in *per* null flies it is not seen as a 'slave' oscillator.

Although larvae of *D. melanogaster* show no obvious rhythmic activity (Sawin et al., 1994), the rhythm of eclosion may be phase set and entrained by light at any stage of post-embryonic development (Brett, 1955). The eclosion pacemaker must therefore operate throughout larval life. Helfrich-Förster (1996) has suggested that lateral neurons (LN_vs) with small somata, which exhibit *per* cycling throughout larval development, may regulate pupal eclosion, whereas all LN_vs, together with the glial cells, may be responsible for locomotor rhythms in the adult fly.

Direct photoreception by the brain was demonstrated by Truman and Riddiford (1970) using the silk moths *Antheraea pernyi* and *Hyalophora cecropia* which, because of their large size, obviated many of the difficulties inherent in *Drosophila*. In a now 'classical' series of experiments, these authors showed that adults of *H. cecropia* emerged in a well-defined 'gate' during the forenoon, whereas adults of *A. pernyi* emerged towards the end of the light-phase

(see Fig. 3.7). Section of the optic nerves, or extirpation of the compound eye *anlagen* from diapausing pupae, was inconsequential, but removal of the brain also removed the 'gating' control of eclosion and the moths emerged in a random fashion. Response to photoperiod was fully restored, however, merely by re-implanting the excised brain into the abdomen (Fig. 8.7).

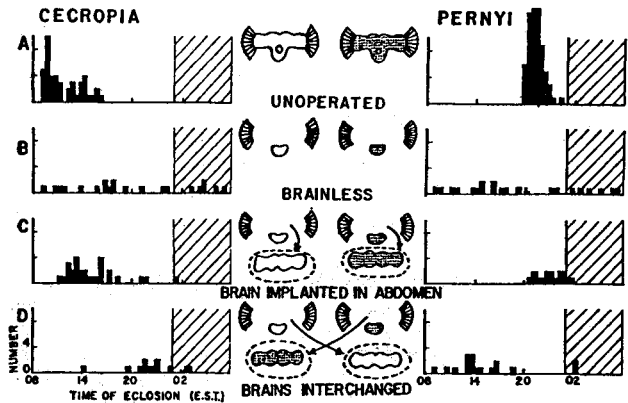


Fig. 8.7. The eclosion of *Hyalophora cecropia* and *Antheraea pernyi* in an LD 17:7 regime showing the effects of brain removal, the transplantation of the brain to the abdomen, and the interchange of brains between the two species. (From Truman, 1971d).

The location of the photoreceptors within the brain was demonstrated unequivocally by removing the brains from twenty pupae of *H. cecropia* and re-implanting them into either the head (ten insects) or the abdomen (ten insects). These 'loose-brain' pupae were then inserted into holes drilled in an opaque board in such a way that the anterior and posterior ends of the pupae received light regimes (LD 12:12) differing only in phase. Figure 8.8 shows that the time of eclosion was determined by the photoperiod to which the brain was exposed.

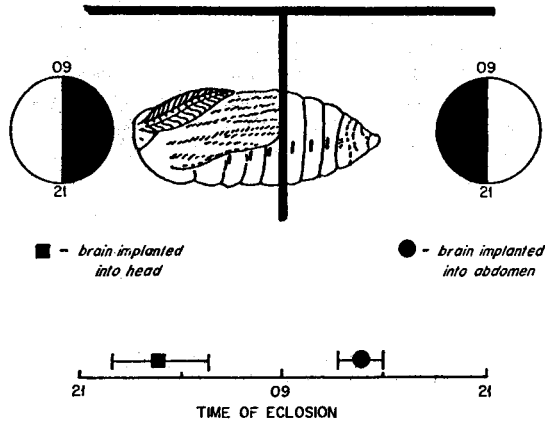


Fig. 8.8. The eclosion of two groups of 'loose-brain' *Hyalophora cecropia* which differed only in the site of brain implantation. The anterior end of each was exposed to light from 09.00 to 21.00; the posterior ends from 21.00 to 09.00. The time of eclosion was determined solely by the photoperiod to which the brain was exposed. The mean and standard deviation of each group is given. (From Truman, 1971d.)

Although the compound eyes are not the photoreceptors for the entrainment of the eclosion rhythm, they are involved in certain exogenous effects caused by the lights-on stimulus (Truman, 1971d). For example, the eclosion peak of *H. cecropia* showed a strong skew towards lights-on that was eliminated after transection of the optic nerves or by the transfer of the brain to the abdomen. When the brain was transplanted together with its attached eye discs, however, well-formed compound eyes developed from these discs, and the immediate response to light was restored.

Truman and Riddiford (1970) also demonstrated that when the brains of *H. cecropia* and *A. pernyi* pupae were interchanged the timing of eclosion was characteristic for the species of *brain*, but the emergence behaviour was characteristic for the *body* of the recipient. Section of the circum-oesophageal connectives, or extirpation of the sub-oesophageal ganglion, frontal ganglion, or *corpora allata-corpora cardiaca* complex had no effect. These very elegant results demonstrated that the clock was also located in the brain and, since 'loose-brain' insects functioned in the same way as intact animals, the output of the clock was hormonal. Furthermore, the hormone involved was neither species nor genus-specific.

In a later paper (Truman, 1972b) attempts were made to locate the clock within the brain itself. Surgical bisection of the brain had no effect on gating control. Brains were then excised from *A. pernyi* pupae and the small central wedge containing the median neurosecretory cells separated from the peripheral areas containing the lateral cells. These pieces were then implanted separately into brainless recipients. In all cases eclosion was random suggesting that neither part of the brain was competent to control the oscillation by itself. Truman pointed out, however, that since each insect emerges only once, one could not discriminate between the result of implantation of a fragment lacking a clock and that of one which just lacked a photoreceptor and was, therefore, free-running. In both instances a randomised distribution of eclosions would result. The result might also be attributed to the damage caused during the surgical procedures.

It was shown that a hormone involved in the control of eclosion (a neurotropic *eclosion hormone*, EH) could be extracted from the brain of *A. pernyi* during the latter two-thirds of pharate adult development (Truman, 1970). It was secreted by the median neurosecretory cells and passed down their axons to the *corpora cardiaca*. Release of this hormone into the blood was a gated event dictated by the eclosion clock. Experimental injection of brain homogenates into moths developmentally competent to emerge, initiated a programme of abdominal movements which led, within two hours, to eclosion, escape from the cocoon and the spreading of the wings. The activation of this nervous programme by the hormone was examined by electro-physiological methods (Truman and Sokolove, 1972). By recording from a nerve cord with severed peripheral nerves it was shown that this pre-eclosion behaviour was pre-patterned in the abdominal ganglia and required no sensory feed-back.

More recent investigations (Ewer et al. 1997) have shown that eclosion behaviour involves two sets of endocrine cells, the centrally located neurosecretory neurons that release EH, and more peripherally located Inka cells that release ecdysis triggering hormone (ETH). Eclosion hormone acts on the Inka cells to release ETH; in turn ETH acts on the EH neurons to cause release of eclosion hormone. This positive feedback leads to reciprocal excitation resulting in massive surges of EH and ETH in the haemolymph, and of EH within the CNS. Eclosion hormone was found to provide a link between ETH and crustacean cardioactive peptide (CCAP) in a neuroendocrine cascade controlling ecdysis behaviour (Gammie and Truman, 1999) (see also Chapter 5).

It is interesting that pharate adults of *A. pernyi* exhibited essentially 'pupal' behaviour until eclosion, restricting their movements to a simple rotation of the abdomen (Truman, 1971

c, d). This pupal behaviour persisted even when the pupal cuticle was removed: 'peeled' moths did not, for example, attempt to spread their wings. When the eclosion gate opened, however, the entire eclosion sequence followed despite the fact that the insects had neither pupal integument nor cocoon to escape from. If the brain was removed this sequence was destroyed. In some insects, for example, eclosion occurred before the moulting fluid was completely resorbed and the moths emerged wet; in others the intersegmental abdominal muscles degenerated prematurely so that the moth was trapped within its pupal cuticle. The proper sequence was restored after a brain, with its clock, was implanted into the abdomen.

In the tobacco hornworm moth, *Manduca sexta*, Truman et al. (1983) showed that a decline in the haemolymph ecdysteroid titre was important for regulating EH release. The steroid drop was shown to be important for two reasons: causing the target tissues to become sensitive to the peptide, and then acting as a hormonal prerequisite for the release of EH itself. This dual action of the declining ecdysteroid titre insured that when EH was released the tissues would be competent to respond. Sauman and Reppert (1996) have mapped EH, PTTH, PDH and PER immunoreactive neurons in the silk moth, *Antheraea pernyi*.

Larval and pupal moults. A circadian clock may also control moulting from one larval instar to the next. Truman (1972a), for example, showed that the larval moults of the moths *Antheraea pernyi* and *Manduca sexta* occurred at particular times of the day depending on the species, the instar involved, and the photoperiod to which the insect was exposed. Figure 8.9 shows that the

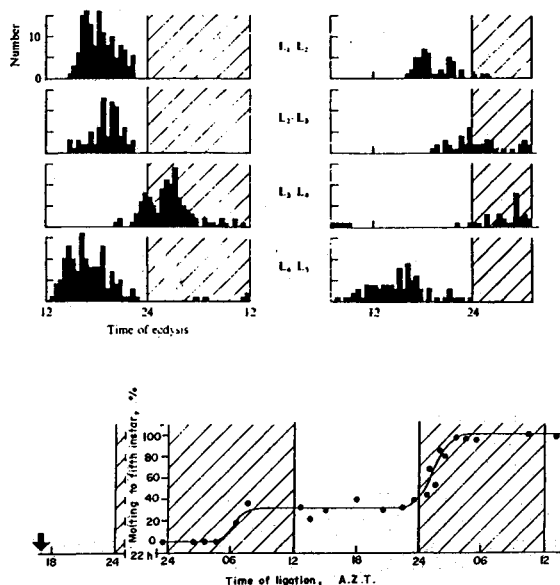


Fig. 8.9 (upper). The distribution of the four larval ecdyses of *Antheraea pernyi* reared at 26°C under (left) an LD 12:12 regime, or (right) an LD 17:7 regime. (From Truman, 1972a.)

Fig. 8.10 (lower). The effects of applying ligatures to fourth instar larvae of *Manduca sexta* at various times after the L₃-L₄ ecdysis. The percentage of the neck-ligated larvae subsequently developing to the fifth instar is plotted against the time of ligation. Each point represents a sample of about fifty ligated larvae. The arrow identifies the median time of the L₃-L₄ ecdysis. (From Truman, 1972a.)

times of the larval ecdyses of *A. pernyi* occurred later in each succeeding instar and that the distributions of ecdysis time tended to broaden. The moult from first to second instar (L_1 to L_2) and from L_2 to L_3 , for example, occurred during the light phases of both LD 12:12 and LD 17:7. The moult from L_3 to L_4 , however, occurred during the following *night*, and that from L_4 to L_5 was delayed until the light phase of the next day. A series of experiments using ligatures between the head and thorax at various times during the light-cycle suggested that the release of brain hormone (prothoracicotropic hormone, PTTH) was a gated event. In *M. sexta*, PTTH secretion was reported to occur only during a gate in the early to middle portion of the night. (Fig. 8.10) (but see Chapter 5, C1 for a more detailed account of these experiments, and an interpretation based on actual hormone measurements showing that PTTH release is not confined to these times). The larval ecdyses themselves were not gated, but occurred a fixed number of hours after the hormonal release. This time interval was instar specific and dependent on temperature, a 3°C rise (25 to 28°), causing a 2-hour advance in the L_3 to L_4 moult. The proportion of the larval population exploiting a particular gate was also temperature-dependent, as in *D. pseudoobscura* and other gated rhythms (see Chapter 3). As with pupal eclosion, the photoreceptors regulating PTTH release are probably located within the brain, caution of the larval ocelli (stemmata) failing to abolish the insects' ability to 'see' the light regime.

Truman's reports on larval moulting were soon followed by studies on the saturniid, *Samia cynthia ricini* (Fujishita and Ishizaki, 1981) and the commercial silk moth, *Bombyx mori* (Sakurai, 1983). Using similarly timed ligatures applied between the head and thorax, both of these studies suggested that release of PTTH was under 'clock' control (see also Chapter 5). As larvae of *Samia* kept under LD 12:12 approached their last (L_4 to L_5) moult, PTTH release was thought to occur in short pulses during the second and third nights of their fourth instar. Under continuous darkness, these pulses occurred $15 \pm \pi$ hours after the clock had started. Although only two pulses of PTTH release were thought to occur, this rhythm was very similar to that described for pupal eclosion in *D. pseudoobscura* (Pittendrigh, 1966; see Chapter 2). Thus, in DD free-run, the period of the rhythm (τ) was slightly less than 24 hours, the rhythm could be phase set and entrained by light, but continuous light led to arrhythmic moulting. The interval between the postulated PTTH peaks was also temperature-compensated, although, as in *D. pseudoobscura*, PTTH release occupied earlier 'gates' at the highest temperature. Secretion of ecdysteroid moulting hormones occurred about 6 hours after PTTH release, and ecdysis to the fifth instar some 34 hours later. In fourth instar larvae of *B. mori* maintained in LD 12:12, gated PTTH releases occurred during the second and third nights (Sakurai, 1983). The second of these pulses caused the prothoracic glands to initiate ecdysone secretion, and the moult to the fifth instar occurred about 11 hours later. The titre of juvenile hormone (JH) was relatively high before the second PTTH release; it then fell and the *corpora allata* became dispensable.

In these moths a circadian clock also controls the moult from larva to pupa. Toward the end of the final larval instar, larvae leave their food plant and prepare for transformation to the pupa. In silk moths (Saturniidae) this process includes the spinning of a silken cocoon; hawk moths, such as *Manduca sexta*, burrow beneath the surface of the soil. A characteristic sign, or *prodrome*, of the approaching larval-pupal transformation is a violent evacuation of the rectum, called the 'gut purge'. Several papers have shown that gut purge and pupation are gated events, and have explored the hormonal correlates of these phenomena during the metamorphosis.

In the silk moth *Samia cynthia ricini*, Fujishita and Ishizaki (1982) and Fujishita et al. (1982) applied carefully timed ligatures between head and thorax to determine the times of PTTH and ecdysteroid release. Under a photocycle of LD 12:12, PTTH release was considered to occur in two 'steps'. During the second or third photophases after the last (L_4 to L_5) moult,

PTTH was released which led, within 1.5 days, to an increased synthesis of ecdysteroids by the prothoracic glands during the scotophase preceding gut purge. PTTH was released for a second time two days after gut purge; this led to the formation of the pupal integument and the moult. Sakurai (1984) showed that final instar larvae of the commercial silk moth *Bombyx mori* underwent gut purge on the sixth to seventh day and then pupated four to five days later. The titre of juvenile hormone (JH) was high on day 1 but then declined to an undetectable level by day 3. PTTH release began after this JH decline and was completed during the night of day 4; from this point the brain was no longer required for the completion of metamorphosis. After stimulation by PTTH, the prothoracic glands secreted ecdysteroids on day 8, leading to gut purge and pupation.

One of the more important papers in this area of enquiry was that by Mizoguchi and Ishizaki (1982) on clock and photoreceptor location in *Samia cynthia ricini*. In this study, fifth instar larvae were transferred from LD 12:12 to DD (to start the clock; see Chapter 3), four days after the last (L_4 to L_5) moult. They were then placed in an apparatus so arranged that light-tight partitions were placed at the neck, between pro- and mesothorax, between meso- and metathorax, or between metathorax and abdomen. Compartments to the anterior or posterior of these partitions were then illuminated, three days later, by a 15 minute light pulse, and the larvae subsequently examined for any delay phase-shift in the time of gut purge. Phase-delays, similar to those observed in whole-body illuminated controls, were only recorded when compartments containing the pro- and mesothorax were illuminated. Illumination of the head alone, or that portion of the body behind the mesothorax, failed to elicit a significant phase delay, indicating the presence of a light-sensitive circadian clock in the pro- and/or mesothoracic part of the thorax. In subsequent experiments, prothoracic glands were removed from donor larvae raised in LD 12:12 and implanted into the abdomens of recipients, to produce larvae with transplanted 'abdominal' glands as well as their own in the thorax. Control larvae received abdominal implants of brain, mesothoracic ganglion or fat body tissue. After transfer to DD, the larvae were illuminated with a 10 second light pulse, either to the anterior portion of the body or to the whole larva. Results showed that when the anterior portions of control larvae were illuminated, a delay in gut purge was recorded. Similar delays occurred in larvae with implants of 'control' tissue, but did not occur when the transplanted tissue comprised prothoracic glands. The conclusion was that when the implanted PGs were not illuminated they produced ecdysteroids 'on time' This secretion led to normal gut purge.

These important experiments showed that in addition to a clock in the brain that regulates PTTH release (Fujishita and Ishizaki, 1982) there is also a light-sensitive circadian clock in the prothoracic glands that 'gates' ecdysone release and the final timing of gut purge. In some respects the brain clock resembles a 'pacemaker' and the PG clock a 'slave' (see Chapter 3), albeit a photosensitive slave. This and other examples of photoreceptive clocks in brain and PGs, notably that in the blood-sucking bug *Rhodnius prolixus* (Vafopoulou and Steel, 1996, 1998), will be described elsewhere (see Chapter 5).

ANNOTATED SUMMARY

1. The photoreceptors for entrainment of cockroach locomotor rhythms are the *compound eyes*. Occlusion, surgical destruction or isolation of these organs causes the rhythmic activity to 'free-run' in LL or LD as though the insects were in DD. The role of the ocelli and/or extra-optic and extra-cephalic photoreceptors is not satisfactorily established, or is still open to question.

2. The 'clocks' controlling locomotor rhythms in cockroaches are in the *optic lobes*. Bilateral section of the optic nerves causes these insects to free-run in LD suggesting that the photoreceptors (compound eyes) have been isolated from the clock. Cutting between the optic lobes and the rest of the brain, however, causes them to become arrhythmic, suggesting that the clock has been isolated from the thoracic locomotory centres.
3. Isolated optic lobes of the cockroach *Leucophaea maderae* support robust rhythms of neural activity when maintained *in vitro*. Transplantation of such an optic lobe to a lobeless recipient also transfers period and phase characteristic of the donor cockroach, after regeneration of the appropriate neural connections.
4. Immunocytochemistry using an antiserum raised against crustacean *pigment dispersing hormone* (PDH) reveals an extensive neuronal architecture that fulfils many of the criteria for a circadian pacemaker. In cockroaches, crickets and other insects, pacemaker neurons are in an area of the optic lobe called the *accessory medulla*, close to the base of the medulla itself.
5. The output from the locomotor clock in cockroaches appears to be nervous. Cutting the circum-oesophageal commissures (in *Leucophaea maderae*) causes arrhythmia which would not be expected if the output was humoral. Neurosecretory cells in the sub-oesophageal ganglion are not the locus of the driving oscillation. The cockroach brain contains two optic lobe clocks because of bilateral symmetry; these clocks are mutually coupled via the *pars intercerebralis*.
6. In crickets, the compound eyes are the most important photoreceptors for entrainment of locomotor or stridulatory rhythms. The circadian pacemakers are in the optic lobes (*accessory medulla*), although there is also evidence for pacemakers in the central brain. Beetles also possess optic lobe pacemakers and, in addition to the compound eyes, have non-visual photoreceptors deep within the head capsule that might be involved in entrainment.
7. Flies probably use multiple photoreceptors for entrainment, but the compound eyes (and ocelli) are *not essential*; there is evidence for extra-optic or brain photoreception. In *Drosophila melanogaster*, neurons close to the border of the optic lobes and central brain (the *lateral neurons*) have been identified as pacemaker cells. In moths, compound eyes and optic lobes are both dispensable, photoreception and pacemaker function lying in the central brain.
8. Entrainment of the *eclosion rhythm* in *Drosophila* spp and silk moths uses photoreceptors in the brain; 'organised' photoreceptors, such as eyes and ocelli, are not essential. Transfer of the pupal silk moth brain from the head to the abdomen also transfers sensitivity to the environmental light-cycle. Certain exogenous effects, however, are mediated through the compound eyes.
9. Removal of the brain from silk moth pupae did not prevent eclosion but removed its gated control. Transfer of the excised brain into the abdomen of the same individual, or into the abdomen of a decerebrated individual of another species, restored the gated control of eclosion, and in the latter case also determined the time of eclosion which was characteristic of the brain of the donor species. Since all nerves are cut in these operations the output from the cerebral clock must be humoral rather than nervous.
10. In silk moths, the circadian clock dictates the time of release of *eclosion hormone* (EH). Experimental injection of brain homogenates into moths competent to emerge initiates a behavioural sequence, characteristic of the species of body, culminating in emergence from the pupal cuticle and cocoon and the spreading of the wings. This sequence is pre-patterned in the CNS. Two other hormones, *eclosion triggering hormone* (ETH) and *crustacean*

cardioactive peptide (CCAP), are also involved in the regulation of eclosion and ecdysis behaviour.

11. In moths, larval to larval moults, and the transformation of larva to pupa, are controlled by a brain-centred circadian clock controlling the release of *prothoracicotropic hormone* (PTTH). The brain and the prothoracic glands may be photoreceptive and contain circadian pacemakers.

CHAPTER 9

PHOTOPERIODISM AND SEASONAL CYCLES OF DEVELOPMENT

In the bleak midwinter/Frosty wind made moan/Earth stood still as iron/Water like a stone.
Christina Rossetti

CONTENTS

Introduction	271
A. <i>Dormancy: Quiescence and Diapause</i>	273
1. Quiescence	273
2. Diapause	273
(a) Types of diapause	274
(b) Summer diapause	275
(c) Diapause in the tropics	275
B. <i>Endocrinology of Diapause</i>	276
(a) Larval and pupal diapause	276
(b) Adult (reproductive) diapause	283
(c) Egg (embryonic) diapause	284
(d) Other types of diapause	285
C. <i>Cold Tolerance</i>	285
D. <i>Seasonal Morphs</i>	286
E. <i>Growth Rates</i>	292
F. <i>Migration</i>	294
G. <i>Miscellaneous Photoperiodic Phenomena</i>	296
Annotated Summary	297

INTRODUCTION

PHOTOPERIODISM comprises a miscellany of clock phenomena in which organisms distinguish the long days (or short nights) of summer from the short days (or long nights) of autumn and winter, and thereby obtain information on calendar time from the environment. A wide range of organisms, principally higher plants and animals living in a terrestrial environment, use this 'noise-free' information to control various seasonally appropriate switches in metabolism, most of which have a clear functional significance or survival value.

Although a number of botanists in the last decades of the nineteenth and the early part of the twentieth century were aware of some day-length effects on flowering plants (see Cumming, 1971), the phenomenon of *photoperiodism* was first adequately described by Garner

and Allard (1920). They found that many plants, including tobacco, soybean, radish, carrot and lettuce, could only flower and fruit when day-length fell between certain limits; some plants responded to long days, others to short. The Maryland Narrowleaf variety of tobacco (*Nicotiana tabacum*), for example, grew by vegetative means to an 'extraordinary' height at day-lengths down to 12 hours per day, but produced flowers and seeds at LD 7:17. The Biloxi variety of soybean (*Soja max*) was also a short-day plant. Garner and Allard found that the type of growth could be manipulated experimentally by supplementing or curtailing the natural day-length. These observations were soon followed by Marcovitch's (1923, 1924) demonstration that the appearance of seasonal morphs in several species of aphids was similarly controlled. The strawberry root aphid, *Aphis forbesi*, for example, produced sexual forms when the natural day-length was curtailed to 7 hours per day, even at the height of summer. Conversely, long exposure to artificial light in September inhibited the sexual forms and induced viviparous reproduction. Rowan (1926) later demonstrated that day-length could have important effects on reproductive behaviour and physiology of birds. Kogure (1933) and Sabrosky et al. (1933) were the earliest workers concerned with the photoperiodic control of insect diapause. Sabrosky and his collaborators showed that diapause in the grasshopper *Acrydium arenosum* could be averted by exposure to continuous light. Kogure's very extensive investigation showed that the commercial silk moth, *Bombyx mori*, was a short-day insect with a winter diapause in the egg (embryo) and included observations on light-intensity thresholds and the spectral sensitivity of the response. Shortly afterwards, Baker (1935) described the photoperiodic termination of diapause in a number of over-wintering tree-hole mosquitoes and midges.

Since these early papers many of the seasonal activities of animals, including insects, crustacea (e.g. Stross and Hill, 1968), acarina (e.g. Lees, 1953a; Belozarov, 1964), birds, mammals and reptiles have proved to be under a similar type of day-length control. Amongst the insects, the induction and termination of *diapause*, and the control of *seasonal morphs* are probably the most widespread phenomena, and certainly the most extensively studied. A number of other aspects of physiology and behaviour, such as the adoption of winter coloration, deposition of fat, protracted development and migration to hibernation sites are often connected with the induction of the diapause state, and therefore brought about by the same environmental factors.

Since the discovery of photoperiodism about eighty years ago a very large number of scientific papers on the subject have appeared. This is particularly true for the control of diapause in the northern temperature zones (approximately 30° to 60°N), and because of the obvious importance of seasonal cycles in the study of agricultural and other pest species. Danilevskii (1965), for example, was able to list over 100 insect species with such a clock. Beck (1968, 1980) raised this number to about 150, and Saunders (Insect Clocks, second edition, 1982a) to over 320 species in 15 orders. More recently, Nishizuka et al. (1998) stated that diapause responses had been recorded in over 500 species in 17 orders. Danilevskii (1965), Beck (1980) and Saunders (1976, 1982a) provided lists of species showing photoperiodic control. This practice is now abandoned because of the very large number of species involved and the fact that - at least within the temperate zones - insects *lacking* a photoperiodic response are probably exceptions. The absence of knowledge on an insect's diapause or photoperiodic control is simply due to a lack of research effort. This chapter will describe the principal photoperiodic phenomena observed in the insects; the properties of the clock and the nature of the time measurement involved will be dealt with in subsequent chapters.

A. DORMANCY: QUIESCENCE AND DIAPAUSE

Unless insects have evolved some specialised habit or habitat-choice, periods of unfavourable climate demand from them a state of reduced metabolism, or *dormancy*, to enable them to over-winter or withstand a dry season. During the favourable season the insect may produce several successive generations (a multivoltine life cycle) or a single generation per year (univoltine); other species may have a life cycle occupying several years with a number of dormancy periods. In tropical areas insects often show bursts of activity when the rainy season begins and become dormant with the start of the dry season. In temperate latitudes insects characteristically commence activity in the spring and become dormant as the winter approaches. In certain subtropical areas, where the summers are hot and dry and the winters cold or cool, two periods - spring and autumn - may be favourable for growth and activity, and dormancy may intervene in both winter and summer (Masaki, 1980).

Ecologically it is possible to distinguish two types of dormancy: *hibernation* and *aestivation*. The physiological mechanisms controlling dormancy, however, may be quite diverse; *quiescence* and *diapause* are the two most important mechanisms involved (Lees, 1955). Since only the latter appears to be controlled by a photoperiodic clock, quiescence will be dealt with very briefly.

1. *Quiescence*

The chief characteristic of quiescence, as opposed to diapause, is that the state of dormancy is directly imposed by the adverse conditions, and recovery occurs soon after these restrictions are removed. For example, insects in a state of 'cold torpor' resume activity when the temperature rises, and dehydrated larvae continue development when water is supplied.

Dehydration occurs most frequently as a mechanism for aestivation. The Chironomid, *Polypedilum vanderplanki*, for example, breeds in pools of water on exposed rocks in parts of west and east Africa. During the dry season these pools dry up and the larvae become almost totally dehydrated (Hinton, 1951). They can tolerate repeated periods of dehydration and hydration and have remained viable in the dry state for many years. Laboratory experiments (Hinton, 1960) demonstrated a remarkable resistance to extremes of temperature whilst in this condition. Dehydrated larvae, which may contain as little as 8 per cent of moisture, can withstand total immersion in liquid helium, and exposure to 102 to 104°C for short periods without preventing recovery and subsequent development when returned to water. Dried larvae have also recovered after immersion in absolute ethanol for 24 hours, and up to 7 days in pure glycerol. It is probable that many other insects that live and breed in ephemeral aquatic habitats can aestivate in a similar dehydrated condition.

2. *Diapause*

Diapause differs from quiescence in two fundamental ways. Firstly, it is an 'actively induced' state most frequently involving the cessation or alteration of neuroendocrine activity, usually at species-specific points in the insect's life cycle. Secondly, the onset of diapause is brought about by environmental factors which, although signalling the approach of 'unfavourable' conditions, are not, in themselves, adverse. These factors have been called 'token stimuli' (Lees, 1955), the most important of which is photoperiod. Since local populations of an insect respond to day length in the same manner, and enter diapause at the

same developmental stage, this mechanism may also serve to synchronise the seasonal activities of the particular species.

Insects entering an over-wintering diapause frequently show an array of physiological and behavioural states making up a characteristic diapause 'syndrome'. These include a cessation of further development or metamorphosis (but not necessarily feeding or locomotor activity), fat body hypertrophy, storage of distinctive metabolites in the haemolymph, reduced metabolism, acquisition of cold hardiness or resistance to desiccation, and sometimes the construction of specialised hibernacula. Finally, depending on its duration, or the ease by which it may be terminated, a diapause may be regarded as either 'intense' or 'shallow'.

There is now an enormous literature on the various aspects of insect diapause. Happily there are several excellent recent texts, particularly those by Tauber, Tauber and Masaki (1986) and Danks (1987) dealing primarily with the ecological aspects of the phenomenon. After a general account of diapause in this Chapter, the present book will therefore concentrate on insect photoperiodic responses (Chapters 10 and 12), the chronobiological aspects of photoperiodic time measurement (Chapters 11 and 13), and the physiology and anatomical location of the photoreceptors and clocks involved (Chapter 14).

(a) Types of diapause

There have been several attempts to classify insect diapauses. Firstly, they have been distinguished according to the stage in the life cycle in which the arrest occurs (e.g. embryonic, larval, pupal or adult; see below). Another criterion has been whether the diapause is considered obligatory or facultative, with the latter usually induced by environmental, particularly photoperiodic, factors. Among the latter has been a useful distinction whether the stage sensitive to environmental stimuli simply precedes the diapause, or extends through it. Unfortunately, some of these distinguishing criteria have appeared to be a product of insufficient research effort, and with increasing information have proved to be less prescriptive than first thought. The classification suggested by Müller (1970), however, still has some currency.

Müller recognised three types of diapause. (1) *Parapause* is an obligatory diapause observed in univoltine species. There is a clearly defined phase of induction and the diapause supervenes in every generation in a species-specific instar. The onset of this type of diapause is genetically determined and appears to be independent of the environment. (2) *Eudiapause* is a facultative cessation of development with species-specific sensitive periods (inductive phases) and diapausing instars. In favourable conditions development proceeds unchecked; as unfavourable seasons approach diapause supervenes. This type of diapause is usually induced by photoperiod, but terminated by a period of chilling (over winter), or by a change in the level of the temperature. (3) *Oligopause* is also a facultative arrest but with photoperiodic sensitivity extending to include the diapause stage; induction *and* termination of diapause may be under photoperiodic control. Mansingh (1971) and Thiele (1973) further developed and discussed this classification.

Insects entering winter diapause generally react to short day length, decreasing day length, or to a change from long to short days. By analogy with plants these insects are called long-day species because they are active during the long days of summer and become dormant in the autumn. Those insects that aestivate, or are winter-active, usually show an opposite, or short-day response (see below). One notable exception to this is the commercial silk moth *Bombyx mori*. Some strains of this moth are short-day insects but overwinter as an embryo within the egg because the stages sensitive to day length (the eggs and young larvae of the

maternal generation) occur in the height of summer when the days are long (Kogure, 1933). These reactions to day length will be considered in detail in Chapter 10.

In some insects such as *Dendrolimus pini* (Geispits, 1965), larvae may enter diapause during one or more of the larval instars, and some long-lived insects, such as the dragonfly *Tetragoneuria cynosura* (Lutz and Jenner, 1964), may diapause as early nymphs in one winter and mature nymphs in the next. The pitcher plant mosquito *Wyeomyia smithii* may diapause in either third or fourth larval instars (Lounibos and Bradshaw, 1975). Other species, such as *Mamestra brassicae* (Masaki and Sakai, 1965), *Hyphantria cunea* (Umeya and Masaki, 1969) and certain carabid beetles (Thiele, 1969), may hibernate and aestivate, sometimes in different instars. The majority of insects, however, enter diapause at a single species-specific point in their life cycle. The silkworm *Bombyx mori* (Kogure, 1933), the mosquito *Aedes togoi* (Vinogradova, 1960) and the green vetch aphid *Megoura viciae* (Lees, 1959), for example, diapause as eggs. A vast assemblage of species enters diapause as larvae or nymphae. These include the pink boll worm *Pectinophora gossypiella* (Adkisson, Bell and Wellso, 1963), the European corn borer *Ostrinia nubilalis* (Beck and Hanec, 1960), the parasitic wasp *Nasonia vitripennis* (Saunders, 1965 a, b), the cricket *Gryllus campestris* (Fuzeau-Braesch, 1966) and the blow flies *Lucilia caesar* (Ring, 1967) and *Calliphora vicina* (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a). Pupal diapause is particularly common in the Lepidoptera, such as *Acronycta rumicis* (Danilveskii, 1965), *Pieris rapae* (Barker et al., 1963) and *Antheraea pernyi* (Tanaka, 1950), and in the Diptera, such as *Erioischia brassicae* (Hughes, 1960), *Lyperosia irritans* (Depner, 1962) and *Sarcophaga argyrostoma* (Fraenkel and Hsiao, 1968). Adult or reproductive diapause is also widespread: examples include *Musca autumnalis* (Stoffolano and Matthyse, 1967), many species of *Drosophila* (Lumme, 1978), the Colorado potato beetle *Leptinotarsa decemlineata* (De Wilde and De Boer, 1961), the heteropteran bug *Pyrrhocoris apterus* (Hodek, 1968; Saunders, 1983) and various coccinellid beetles (Hodek and Cerkasov, 1958).

(b) Summer diapause

Summer diapause, or aestivation, is commonly thought to occur in areas with a hot and dry summer, the 'active' stages of the life cycle being restricted to the cooler and wetter seasons. However, although it may predominate in such areas - where food plants may perish during the summer - it has been recorded from a wide range of habitats up to 60°N. Masaki (1980) recorded 183 examples of summer diapause from 12 orders of insects (and Acari) where it occurred in the egg, larva, pupa or adult. Masaki defined summer diapause as a form of dormancy induced before the height of summer, and terminated and followed by reproduction, development and feeding in the autumn or winter. In many ways it is a 'mirror image' of winter diapause. It is induced by long photoperiod in conjunction with high temperature, and prevented or terminated by short photoperiod and lower temperature. Species showing a summer diapause are therefore most frequently *short-day species*, since they are metabolically 'active' during the shorter days of autumn, winter or spring (see Chapter 10). Finally, as in the case of winter diapause, insects entering a summer diapause frequently show fat body hypertrophy, reduced metabolism and an increased resistance to desiccation.

(c) Diapause in the tropics

Insects in tropical environments (roughly 20°N to 20°S) may exhibit seasonal cycles of dormancy and metabolic activity, although it is not always clear from the available evidence

whether such dormancies represent 'true' diapause or merely quiescence. Within the tropics seasonal changes in day length are much reduced (about 2 hours and 25 minutes at 20°N or S) and, of course, virtually non-existent at the equator. However, many areas have pronounced seasonal changes in climate, often with one or two dry seasons and associated changes in vegetation, flooding and fires. These and other biotic factors may be important in an insect's abundance.

Denlinger (1986) recorded dormancy in about 73 species across six orders of insects. Dormancy occurred at any stage in the life cycle: egg, larva, pupa or adult. Photoperiodically regulated diapause may be frequent (although by no means universal) at the higher latitudes within this range. Even at lower latitudes (e.g. 7°N to 7°S) such control may be evident, for example, in the red desert locust *Nomadacris septemfasciata* (Norris, 1965; see Chapter 10) and the meal moth *Plodia interpunctella* (Bell et al., 1979). Closer to the equator photoperiodic control may be lost.

Two studies on tropical diapause stand out from the rest. Working at Nairobi, Kenya (1°S), where annual changes in day length are only ± 7 minutes, Denlinger (1974) showed that a pupal diapause occurred in several species of flesh flies (Sarcophagidae). Induction of diapause was shown to depend on temperature, not photoperiod, with larval exposure to temperatures below about 18°C being the most effective. In East Africa such cooler conditions led to the occurrence of diapause in July and August. This investigation was later extended to include Sarcophagids from African localities up to 9°N and to *Sarcophaga ruficornis* from Belem, Brazil (1°S). No effect of photoperiod on diapause induction could be demonstrated; cool temperatures during larval development were the most effective stimuli.

In contrast, a study of the fungus beetle *Stenotarsus rotundus* on Barro Colorado Island, Panama (9°N) revealed a photoperiodic control of its reproductive diapause. Wolda and Denlinger (1984) showed that adults of *S. rotundus* formed dense aggregations of up to 70,000 diapausing adults on a single tree. The beetles remained in diapause for up to 10 months of the year showing characteristic signs of a 'true' diapause: reduced metabolism, fat body hypertrophy, resistance to desiccation and degeneration of the flight muscles. In a series of papers, Tanaka et al. (1987a, b; 1988) demonstrated that seasonal change in photoperiod, even at this latitude, was the major environmental factor involved in diapause induction and termination. From June to September some of the beetles developed their gonads in response to long days (LD 13:11) whereas no such development occurred at LD 12:12. From October to December, beetles showed no rapid gonadal development at either photoperiod, but between January and April gonadal and flight muscle development occurred rapidly under long days. Beetles thus responded to photoperiod throughout the season, leading to reproductive activity during the relatively long days from mid-April to July. Migration from the diapausing aggregations to feeding sites began before the summer solstice.

Although *S. rotundus* showed clear photoperiodic sensitivity, the seed bug *Jadera aeola* and flesh flies living in the same area, were photoperiodically neutral (Tanaka et al., 1987a). Similarly, on Guadeloupe, West Indies (16°N), facultative larval diapause in the parasitic wasp *Cothonaspis bouldardi* was independent of photoperiod (Claret and Carton, 1980).

B. ENDOCRINOLOGY OF DIAPAUSE

(a) Larval and pupal diapause

The hormonal regulation of insect diapause has been extensively reviewed on a number of occasions (see for example, Denlinger, 1985; Saunders, 2000a).

In many species, larval, pupal (and nymphal diapause) is regarded as an inactivation of the brain-prothoracic gland axis (Williams, 1952; Novak, 1966). Under the appropriate photoperiodic influence, the neurosecretory cells of the brain fail to release the brain or prothoracicotropic hormone (PTTH), thus the prothoracic glands remain inactive and, with the resulting low titre of ecdysteroids, growth and development stop (Fig. 9.1). This form of control has been established or postulated for *Hyalophora cecropia* (Williams, 1946, 1952), *Cephus cinctus* (Church, 1955), *Leuodorphia japonica* (Ichikawa and Nishiitsutsuji-Uwo, 1955), *Mimas tilae* (Highnam, 1958), *Ostrinia nubilalis* (Cloutier et al., 1962), *Lucilia caesar* (Fraser, 1960), *Pieris rapae* (Kono, 1973) and *Sarcophaga* spp. (Zdarek and Denlinger, 1975). Several more recent papers have addressed the problem of the hormonal regulation of pupal and larval diapause, especially in the Lepidoptera and Diptera. These studies will be reviewed here.

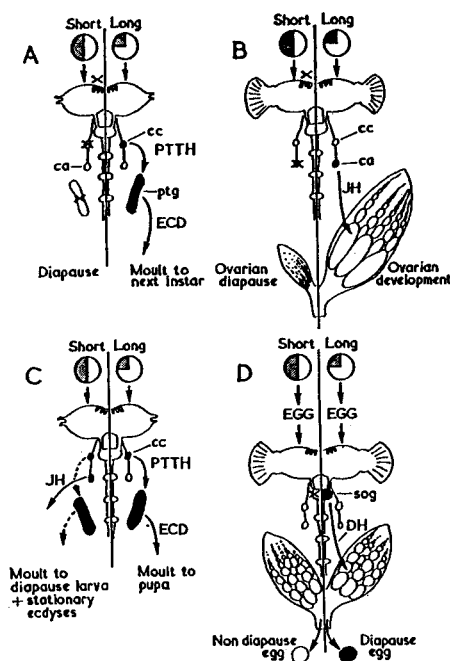


Fig. 9.1. Four types of diapause in insects (schematic). A – Larval-pupal diapause in which short days inactivate the neurosecretory cells of the brain. This results in inactive prothoracic glands, a low titre of ecdysteroids, and diapause supervening before the next moult. Long days allow brain hormone release, elevated ecdysteroid synthesis and moulting to the next instar (after Williams, 1952). B – Adult (ovarian or reproductive) diapause in which short days inactivate the brain neurosecretory cells and hence the corpora allata. The consequent low titre of juvenile hormone (JH) causes yolk deposition in the oocytes to cease. Long days allow ovarian development. In ovarian diapause metabolites are frequently redirected to the fat body as the insect enters diapause (after De Wilde, 1959). C – Larval diapause in *Diatraea grandiosella* in which the brain-prothoracic gland axis remains active under short days, but a reduced titre of JH leads to a series of stationary ecdyses. Long days cause a greater fall in the titre of JH and the larva pupates (after Yin and Chippendale, 1973). D – Embryonic (egg) diapause in *Bombyx mori*. Long days perceived by the eggs and young larvae of the maternal generation result in the secretion of a diapause hormone (DH) by the pupal sub-oesophageal ganglion that enters the ovarian egg and imposes the diapause state (after Fukuda, 1963). Active glands are shown in black or shaded, inactive glands are shown with a cross. cc – corpora cardiaca; ca – corpora allata; ptg – prothoracic glands; sog – suboesophageal ganglion. PTTH – prothoracicotropic (brain) hormone; ECD – ecdysteroids; JH – juvenile hormone; DH – diapause hormone.

Lepidoptera. Working with the tobacco horn worm moth, *Manduca sexta*, Bowen et al. (1984, 1985) investigated interactions between the brain and the prothoracic glands (PG) in the regulation of *pupal* diapause. Determinations of haemolymph ecdysteroid titres by radio-immunoassay (RIA) showed no obvious differences between diapause-destined (short day) and nondiapauses-destined (long day) insects through the 5th larval instar, larval wandering and pupation. Therefore, despite the fact that photoperiodic sensitivity commenced as far back as the first larval instar, no significant differences in ecdysteroid titres were detected. In diapausing pupae, however, the ecdysteroid titre fell to a very low level, whereas in nondiapausing animals it rose to a broad sustained peak associated with the development of the pharate adult (Fig. 9.2). *In vitro* ecdysone synthesis by excised PG was also studied. Glands from nondiapausing pupae exhibited high ecdysone synthesis, while diapausing PG synthesis was markedly low. Furthermore, experiments in which PG were exposed to *in vitro* PTTH exposure showed that glands from diapausing pupae were almost completely refractory to PTTH stimulation by day 15 of pupal life. Since the PTTH content of diapausing brains was similar to that of nondiapausing brains, it was concluded that the low diapausing pupal ecdysteroid titre, and hence diapause, was a result of a curtailment of PTTH release and a refractoriness of the prothoracic glands to PTTH stimulation. A similar hormonal mechanism was observed in the pupal diapauses of *Mamestra brassicae* (Agui, 1975), *Pieris brassicae* (Calvez, 1976) and *Heliothis virescens* (Loeb, 1982).

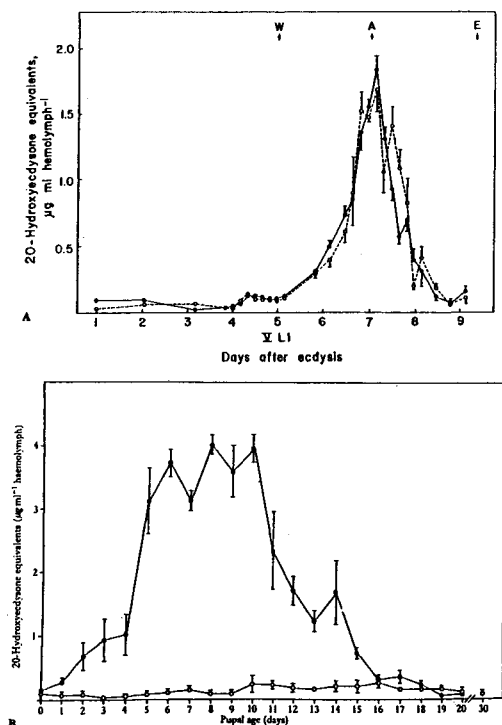


Fig. 9.2. *Manduca sexta*, pupal diapause. Haemolymph ecdysteroid titres (A) during the last larval instar, and (B) during the pupal instar, of insects reared under short days (open circles, LD 12:12, diapause destined) or under long days (closed circles, LD 16:8, nondiapauses destined). Each datum point represents mean \pm SEM of 4 to 8 animals. W – wandering stage; A – apolysis; E – pupal ecdysis. Data from Bowen et al. (1984a, 1985).

Several studies on the termination or prevention of pupal diapause in Lepidoptera by chemical or hormonal means may provide important insights into control mechanisms, particularly those concerning the early stages preceding the release of neurosecretory material from the brain. Rasenick et al. (1976), for example, demonstrated a six-fold increase in brain cyclic AMP in chilled (i.e. reactivated) pupae of *Hyalophora cecropia*, and showed that injection of dibutyryl cAMP into insufficiently chilled pupae also initiated development. In a later paper, Rasenick et al. (1978) demonstrated a dramatic (three-fold) increase in brain cAMP associated with the activation of neurosecretion in *Antheraea pernyi*, brought about by transferring diapausing pupae from short days (LD 10:14) to long days (LD 17:7). Immunofluorescent techniques determined that this increased cAMP activity was in the median neurosecretory cells of the pars intercerebralis, and the authors concluded that the increase was associated with the 'transduction of the photoperiodic signal'.

Larval diapause in the codling moth, *Laspeyresia pomonella* (Sieber and Benz, 1977, 1980) and the European corn borer, *Ostrinia nubilalis* (Bean and Beck, 1980, 1983) also seems to depend on an inactivation of the brain-PG (PTTH-ecdysone) axis. In neither of these species was a significant role demonstrated for juvenile hormone (JH). The role of the brain in *O. nubilalis* was first examined by Cloutier et al. (1962). These authors showed that larval diapause could be terminated by the implantation of brains from either nondiapausing or diapausing larvae, the latter probably being effective because PTTH was released as a consequence of wounding during the transplant procedure. High levels of PTTH in the brains of diapausing corn borers, and PTTH-refractoriness of diapausing PG, were also later demonstrated by Gelman et al. (1992). These studies indicated that diapausing brains contained as much PTTH activity as those from nondiapausing animals, and that larval diapause in *O. nubilalis* was readily attributable to the non-release of PTTH and a consequent low titre of ecdysteroids in the haemolymph (Fig. 9.3).

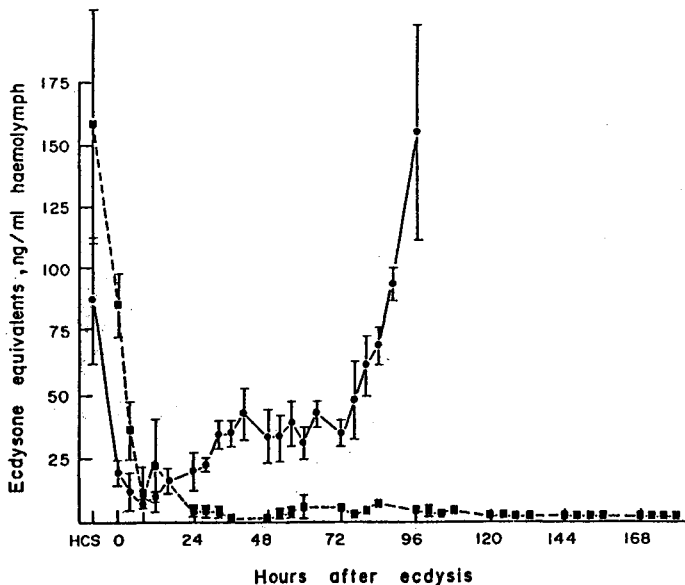


Fig. 9.3. *Ostrinia nubilalis*, larval diapause. Haemolymph ecdysteroid titres during the last larval stadium in prediapause (closed squares) and nondiapause (closed circles) larvae. Each datum point represents a mean \pm SD of 3 to 5 replicates. Data from Bean and Beck (1983).

In some other examples of larval diapause, however, this 'hormonal failure' theory appears to be inappropriate (Chippendale, 1977; Riddiford and Truman, 1978). In the south-western corn borer, *Diatraea grandiosella*, for example, the diapausing larva apparently retains an active endocrine system throughout its period of dormancy. Chippendale and Yin (1973) and Yin and Chippendale (1973) showed that diapause larvae underwent 'stationary moults' in a diapause-maintaining photoperiod of LD 12:12. All larvae raised in LD 12:12 moulted from a 'spotted' (nondiapause) form to an 'immaculate' (diapause) form. About half of them then underwent a second immaculate-immaculate moult, and about 14 per cent a third. At each ecdysis the head capsule remained the same size and the larvae showed physiological characteristics of the diapause state: they did not feed, they had a low respiratory quotient, partial dehydration, increased cold hardiness and accumulation of fat. Injection of ecdysone (20-hydroxyecdysone) into diapausing larvae caused further immaculate-immaculate moults. Neck ligation caused a premature termination of diapause and pupation of the thoraco-abdominal compartment, showing that a cephalic factor was necessary for the maintenance of diapause. Neck ligation and subsequent injection of ecdysone into the body caused premature pupation of that part behind the ligature, showing that ecdysone only caused diapause termination in the absence of this cephalic factor. Topical application of juvenile hormone (JH) caused nondiapause larvae to become immaculate and enter diapause, and repeated application of JH prolonged diapause and increased the number of stationary ecdyses. The cephalic factor that maintains diapause in *D. grandiosella* was therefore identified as juvenile hormone (Fig. 9.4).



Fig. 9.4. *Diatraea grandiosella*, larval diapause. Haemolymph juvenile hormone titres in relation to the onset of diapause and the rate of post-diapause pupation. JH titres measured by the *Galleria* wax test (1 *Galleria* unit ~ 2.6 pg JH equivalents). Ecdysis from a spotted (nondiapause) to an 'immaculate' (diapause) morph (at about day 50) shows that diapause has begun. Data from Chippendale (1984).

Similar stationary larval moults have been described for other Lepidoptera including *Diatraea lineolata* (Kevan, 1944), *Cohiesta ignefusalis* (Harris, 1962), *Busseola fusca* (Usua, 1973) and *Spilarctia imparilis* (Sugiki and Masaki, 1972). In the rice-stem borer *Chilo suppressalis*, juvenile hormone was also shown to be the key factor in inducing and maintaining larval diapause (Yagi and Fukaya, 1974). Even under long day-length, or when diapause was normally terminated by chilling at 5°C for 40 days, application of juvenile hormone re-induced the diapause state.

Diptera. The 'higher' Diptera (Cyclorrhapha) differ from the Lepidoptera in that the third instar larval integument is retained as a hardened puparium around the pupa (Fraenkel and

Bhaskaran, 1973), and the various endocrine glands (corpora cardiaca, corpora allata and the prothoracic glands) are contained within a single structure, the *ring gland* (RG), behind the brain. Information on the endocrine regulation of pupal diapause in this group is available for flesh flies (*Sarcophaga* spp.), which show an embryonic sensitivity to photoperiod (Chapter 10). Short days experienced by the intra-uterine embryos and young feeding larvae thus lead to pupal diapause which supervenes, within the puparium, shortly after pupal head eversion (Fraenkel and Hsiao, 1968a).

Haemolymph ecdysteroid titres in diapause- and nondiapause-destined groups of flesh flies were followed in *S. crassipalpis* by Walker and Denlinger (1980) using the *Musca* bioassay (Kaplanis et al., 1966), and in *S. argyrostoma* by Richard et al. (1987) using radioimmunoassay (RIA). In nondiapause-destined animals raised under long days, ecdysteroid titres following pupariation were shown to occur in two peaks, one driving pupariation and pupation, the other driving pharate adult development. In the short day, diapause-destined groups, however, the first pupariation peak was followed by a consistently low titre of ecdysteroids as the pupae entered diapause (Fig. 9.5). *In vitro* experiments with *S. argyrostoma* ring glands were conducted to ascertain whether they could be stimulated by PTTH extracted from pupal brains (Richard and Saunders, 1987). These studies showed that diapausing brains contained as much PTTH as nondiapausing brains, but that ring glands from diapausing pupae lost competence to respond to PTTH about 4 days after pupariation (Fig. 9.6). The low titre of circulating ecdysteroids characteristic of diapausing pupae, therefore, appeared to be a consequence of the non-release of PTTH together with a developing refractoriness of the prothoracic glands.

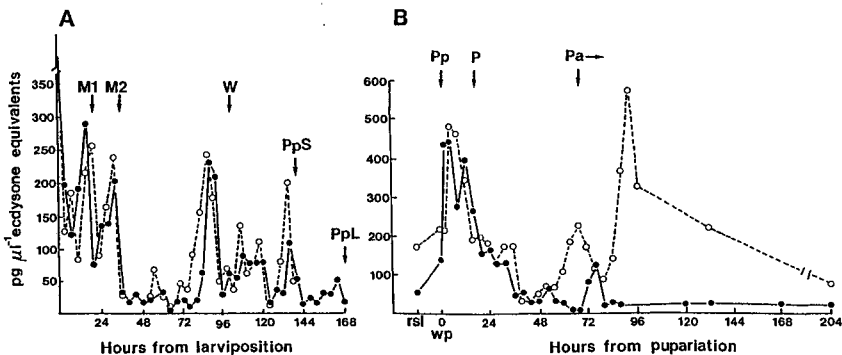


Fig. 9.5. *Sarcophaga argyrostoma*, pupal diapause. Haemolymph ecdysteroid titres of (A) larval and (B) intra-pupal stages taken from diapause destined (closed circles, LD 12:12) and nondiapause destined (open circles, LD 18:6) cultures at 25°C. M1 and M2 – 1st and 2nd larval moults; W – initiation of larval wandering; PpS – pupariation of short night (nondiapause destined) larvae; PpL – pupariation of long night (diapause destined) larvae; Pp – pupariation; P – pupation; Pa – pharate adult development (short nights only); rsl – red spiracled larvae; wp – white puparium. Data from Richard et al. (1987).

Larval diapause in flies is widespread but there is a paucity of data on its endocrine regulation. One of the best-known examples is the blow fly, *Calliphora vicina*. In this species, diapause is largely maternal in origin (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a; see Chapter 10). Autumnal short days experienced by the adult female fly induce her to produce progeny that enter a fairly shallow diapause in the post-fed third instar larva, provided that the temperature falls below about 15°C by the start of larval wandering (Vaz Nunes and

Saunders, 1989). Maternal exposure to long days, on the other hand, leads to nondiapause larvae, pupariation and uninterrupted development to the next generation of flies. As with pupal diapause in *Sarcophaga* spp., a low titre of haemolymph ecdysteroids appeared to be the immediate cause of larval diapause in *C. vicina*. Measured titres were low (Richard and Saunders, 1987), and injections of moulting hormone (20-hydroxyecdysone, 1 μ g) terminated diapause (Maslennikova, 1977). *In vitro* responses of *C. vicina* ring glands to brain extracts indicated that the prothoracic glands became refractory to PTTH stimulation as the larvae entered diapause (Richard and Saunders, 1987).

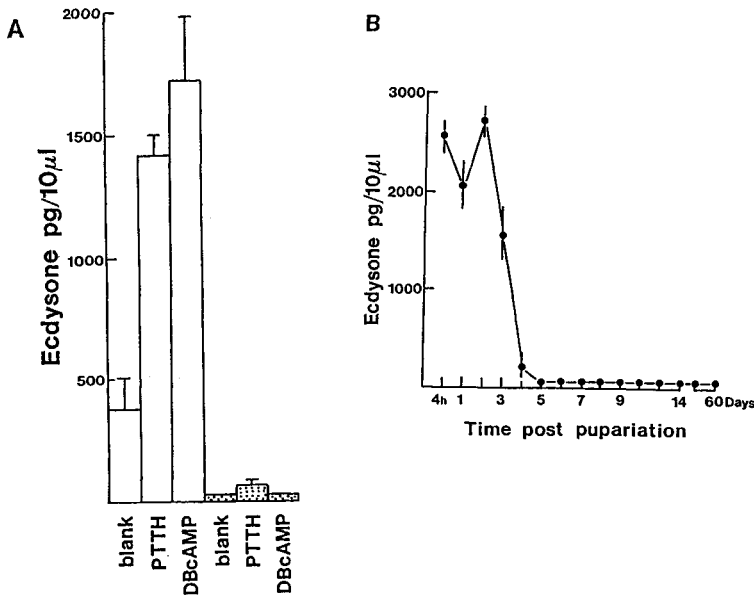


Fig. 9.6. *Sarcophaga argyrostoma*, pupal diapause. A – *in vitro* activation of RGs with PTTH (0.5 brain equivalents) or a cAMP analogue (dibutyl cAMP). Open histograms, RGs from nondiapause destined larvae; stippled histograms, RGs from diapause pupae (2 days after pupariation). B – competency of RGs from diapause-destined pupae to synthesis ecdysone in response to incubation with 0.5 brain equivalents of PTTH prepared from 4-hr prepupal brains. As the pupae enter diapause (days 3 to 4) the RGs become refractory to PTTH stimulation. Data from Richard and Saunders (1987).

In *Sarcophaga crassipalpis*, pupal diapause may be broken by an injection of cyclic GMP and its derivatives, but not by cyclic AMP (Denlinger and Wingard, 1978). Nevertheless, cholera toxin, a stimulant of adenylate cyclase and a potent generator of cAMP, will prevent diapause when injected into diapause-committed larvae 24 hours before puparium-formation (Denlinger, 1976). Cholera toxin is also moderately effective in breaking diapause in the pupa, as are phosphodiesterase inhibitors (caffeine, aminophylline and papaverine) (Denlinger and Wingard, 1978). Cyclic nucleotides may therefore play an important role in breaking diapause and initiating adult development. Diapausing pupae of *S. crassipalpis* (and *Manduca sexta*) are also stimulated to develop by various chemical agents including alkanes, diethyl ether and solvents such as acetone and 2-butanone (Denlinger et al., 1980). Since debrained pupae failed to develop after such treatment it was concluded that the brain, rather than the prothoracic

glands, were responding to the stimulation. The chemicals are thought to exert their effect by altering membrane permeability within the brain.

(b) Adult (reproductive) diapause

The immediate cause of imaginal or reproductive diapause is the inactivation of the brain neurosecretory cells controlling the *corpora allata*; inactive corpora allata result in an absence of juvenile hormone (JH) and suppression of the ovaries, often at the point when yolk deposition should begin. This type of endocrine control was demonstrated for *Dytiscus marginalis* (Joly, 1945), *Leptinotarsa decemlineata* (De Wilde et al., 1959; De Wilde and de Boer, 1961), *Pyrrhocoris apterus* (Slama, 1964), *Anacridium aegyptium* (Geldiay, 1967) and *Galeruca tanacetii* (Siew, 1965 a, b, c). It is usually accompanied by physiological changes such as the deposition of fat and other metabolites in the fat body rather than in the ovaries (gonotrophic dissociation). Hodkova (1976, 1977) also demonstrated a nervous inhibition of corpus allatum activity in *Pyrrhocoris apterus*. In this insect, short days (LD 12:12) led to an ovarian diapause, whereas normal reproduction occurred in long days (LD 18:6). Cutting the *nervi allati* in long-day conditions had no effect on reproduction. But the same operation disturbed the inhibitory effects of short days, and insects with cut nerves proceeded to mature their ovaries and lay eggs. It was concluded that the *pars intercerebralis* of the brain (presumed to be the location of the photoperiodic clock; see Chapter 14) exerted both stimulatory and inhibitory effects on the corpora allata. Under long days it stimulated the corpora allata via the haemolymph, and the glands responded by producing juvenile hormone. Under short days, however, the *pars intercerebralis* inhibited the corpora allata by a neural channel through the *nervi allati*. A similar control mechanism may operate in the photoperiod-sensitive Kazalinsk strain of *Locusta migratoria migratoria* (Darjo, 1976).

Reproductive diapause frequently occurs in fruit flies (*Drosophila* spp.). Of those species for which data are currently available, 16 overwinter in an ovarian diapause in which short days elicit a block to vitellogenesis; one (*D. deflexa*) over-winters as a larva (Basden, 1954) and another (*D. alpina*) as a pupa (Lumme, 1978). Of those diapausing as an adult, most attention has been paid to *D. phalerata*, *D. littoralis* (Lankinen, 1986a) and *D. auraria* (Pittendrigh and Takamura, 1987). Lumme (1978) reviewed the biology of photoperiodic diapause in a number of drosophilids in northern Europe.

The species most extensively used for genetic analysis – *D. melanogaster* – was for a long time considered to be *day neutral* and without a diapause, probably passing the winter when necessary in a state of cold torpor or quiescence (see for example, Izquierdo, 1991). However, a photoperiodically regulated adult diapause was shown to occur in certain strains of *D. melanogaster* when newly emerged adult flies were transferred to short days at a temperature lower than about 14°C (Saunders et al., 1989; Saunders and Gilbert, 1990). It was shown that female flies (Canton-S strain) in these conditions showed a much reduced juvenile hormone (JH) synthesis by corpora allata maintained *in vitro*, and ovaries which remained small and pre-vitellogenic for up to six weeks. Although yolk was not deposited in the oocytes, these flies showed an accumulation of yolk polypeptides in the haemolymph (Saunders et al. 1990). Under long days at the same temperature, JH synthesis *in vitro* was about four times higher and a slow cycle of vitellogenesis and oviposition occurred. Diapausing flies transferred to long days or an elevated temperature rapidly resumed egg development attesting to the rather shallow nature of the diapause in this species. Vitellogenesis also resumed after topical applications of the native JH (JHB₃) or an analogue (methoprene) (Saunders et al. 1990). Effects of so-called ‘clock’ mutants (*per*^S, *per*^L and *per*^O; see Chapter 4) on the photoperiodic

clock of *D. melanogaster* will be discussed elsewhere (Chapter 13) and a genetic analysis of ovarian diapause in this species has been started (Williams and Sokolowski, 1993).

Male insects may also hibernate or aestivate in a reproductive diapause, and spermatogenesis is often suppressed or arrested during dormancy. In the carabid beetle *Pterostichus nigrity*, for example, the males enter an oligopause under long days. Short days, on the other hand, activate the corpora allata and the gonadotrophic hormone produced promotes the maturation of the testes. The hormone involved is thought to be juvenile hormone (JH); similar effects may be induced experimentally by injection of 10,11-farnesyl-methyl-ester, synthetic JH, or the implantation of active corpora allata into undeveloped (long-day) beetles (Ferenz, 1975).

Orshan and Pener (1979a, b) showed that the grasshopper, *Oedipoda miniata*, passed the long, dry summer in Israel in a reproductive diapause in which the females laid no eggs and the males showed little or no mating behaviour. Shortening autumnal photoperiod terminated diapause and induced male mating activity, whereas long summer days and high temperature maintained diapause. Diapause could be induced, terminated and re-induced repeatedly in the laboratory. In a second acridid species, the grasshopper *Anacridium aegyptium*, Greenfield and Pener (1992) and Pener and Greenfield (1992) showed that male sexual behaviour ceased during the autumn months. In a population from northern Italy, adults were found from October to March but oviposition was delayed until December/January when the rains began. Females showed a long reproductive diapause in the field but remained sexually receptive. Males, however, showed a period of reproductive diapause in which sexual behaviour was not expressed. Active corpora allata were shown to be required for initiation and maintenance of male sexual activity.

Short days also induced male reproductive diapause in the linden bug, *Pyrrhocoris apterus* (Hodkova et al., 1991). Males collected from the field in October, or reared under short days in the laboratory, only resumed sexual activity after about two weeks under long days (LD 18:6). Additional stimulation by the presence of receptive females was observed in some conditions. Active inhibition of male mating behaviour was found to involve a neural pathway from the brain (pars intercerebralis), not passing through the corpora allata (Hodkova, 1994). The pars intercerebralis was therefore shown to mediate the effects of photoperiod on male behaviour.

(c) Egg (embryonic) diapause

Egg (or embryonic) diapause may occur at any stage of embryogenesis (Lees, 1955); some species diapause as fully formed larvae within the eggshell, others are at an earlier stage in their development. In many such cases, the onset of embryonic diapause is determined by the photoperiod experienced maternally (see Chapter 10). In some species (e.g. the commercial silk moth, *Bombyx mori*, and the tussock moth, *Orgyia antiqua*) diapause is imposed on the embryo by a 'diapause hormone' which is produced by the maternal sub-oesophageal ganglion and passed into the ovarian egg.

In bivoltine races of *Bombyx mori*, long days and high temperatures acting upon the eggs and young larvae during the summer cause moths of the next (autumnal) generation to lay diapausing eggs. Conversely, short days and low temperature in the spring lead to a generation of moths laying nondiapausing 'summer' eggs. Early experiments by Fukuda (1951) and Hasegawa (1951) demonstrated that a diapause 'factor', now called the *diapause hormone* (DH), was released from the sub-oesophageal ganglion (SOG) of diapause egg producers and entered the ovary during vitellogenesis. Surgical removal of the SOG (from pupae) eliminated

diapause egg production, whereas transplantation of SOG from diapause producers to nondiapause producers resulted in females laying diapause eggs. Excision of the brain from diapause producers led to a fall in the incidence of diapause, whereas removal of the brain from nondiapause producers led to its increase. Histological evidence (Fukuda and Takeuchi, 1967a, b) suggested that DH was produced by a single pair of neurosecretory cells in the SOG. The brain-SOG axis therefore emerged as the maternal regulator, with the brain postulated to exert neural control of the SOG via the circum-oesophageal connectives.

This, now 'classical', example of embryonic diapause has proceeded to a point where the primary structure of DH has been determined (Imai et al., 1991; Sato et al., 1992). Its role in inducing diapause has also been confirmed by using a polyclonal antibody, raised against a synthetic form of the hormone, to neutralise its effect *in vivo* (Shiomi et al., 1994). The photoreceptors and the location of the brain-centred clock controlling DH release will be described later (Chapter 14).

(d) Other types of diapause

A number of Lepidoptera, including the wild silk moth, *Antheraea yamamai* (Suzuki et al., 1990), the gypsy moth, *Lymantria dispar* (Suzuki et al., 1993), and the skipper *Thymelicus lineola* (McNeil and Fields, 1985), over-winter as fully formed (pharate) first instar larvae within the eggshell. Suzuki et al. (1990, 1993) showed that this type of diapause was regulated by a previously unknown mechanism apparently independent of the 'recognised' endocrine centres. Lee and Denlinger (1996, 1997), however, later showed that a characteristic protein was expressed in the gut of pharate larvae of *L. dispar* during the early stages of diapause, and that ecdysteroids were essential for its production. A drop in the ecdysteroid titre was found to be responsible for its termination. The postulated role for ecdysteroids in this form of diapause was therefore in contrast to the classical 'hormonal failure' theory generally accepted for larval and pupal diapauses in other species (see above): in this case, the *presence* of ecdysteroids has a role in inducing or maintaining the developmental arrest.

A few species of Lepidoptera are known to over-winter as fully formed (pharate) adults within the pupal integument (Munro, 1972; Sahota et al., 1982). In the Douglas-fir cone moth, *Barbara colfaxiana*, for example, diapause may span one or two winters with the cessation of development occurring in the advanced pharate adult close to eclosion (Sahota et al., 1982). The endocrinology of this form of diapause is entirely unknown but is likely to differ from that in pupal diapause. It may, for example, involve a 'block' to the release of eclosion hormone (EH) (Truman et al., 1981; see Chapter 8).

C. COLD TOLERANCE

Insects entering a winter diapause frequently acquire a degree of cold tolerance (Bale, 1987, 1993; Pullen, 1996). In different insects this may range from 'freeze tolerance' to mere 'chill tolerance'; such strategies may include, *inter alia*, a depression of the freezing point, accumulation of various cryoprotectants or thermal hysteresis proteins (see below), or an alteration of body water content. In some cases cold tolerance occurs as a result of prior exposure to reduced temperatures. In others it appears to be an integral part of the 'diapause syndrome' (Denlinger, 1991). It is these latter cases which will be considered here, bearing in mind that many species well known for their cold tolerance have not been examined for diapause, and *vice versa*. Cold tolerance has been extensively reviewed in an excellent multi-author volume on the subject, edited by Lee and Denlinger (1991).

One of the clearest examples of a close association between cold hardiness and diapause is that afforded by flesh flies (Adedokun and Denlinger, 1984). In both *Sarcophaga crassipalpis* and *S. bullata*, diapausing pupae showed a high degree of cold tolerance whereas non-diapausing pupae succumbed to the cold. For example, in *S. crassipalpis*, exposure of the intra-uterine embryos and young feeding larvae, at 20°C, to short days (LD 12:12) produced a mixed population of diapausing and nondiapausing pupae. Most (75 to 80 per cent) of the diapausing pupae survived up to 25 days exposure to a temperature of -10°C, whereas practically all of their nondiapausing siblings, bred under identical conditions, died within one day's exposure. In *S. bullata*, a maternal effect that eliminated diapause from the progeny of females that had undergone diapause themselves (Henrich and Denlinger, 1982; see Chapter 10), also eliminated cold tolerance. Resistance to cold is clearly part of the diapause syndrome in these flies.

A second example of a close association between photoperiodism and cold tolerance is shown by the beetle *Dendroides canadensis*. In this species, Horwath and Duman (1983) demonstrated that larvae exposed to short days (less than about 10 hours per day) showed an increased synthesis of 'antifreeze' proteins, whereas in those exposed to longer days (over 11 hours of light) such synthesis was greatly reduced. The circadian system was shown to be involved in the photoperiodic clock regulating the production of these proteins (Horwath and Duman, 1982; see Chapter 11).

Higuchi and Kimura (1985) showed that adults of *Drosophila auraria* developed cold hardiness to a greater extent under diapause-inducing short days than under nondiapauses-inducing long days. For example, survival after 8 days at -5°C was greater in LD 10:14, particularly with a transfer from the rearing temperature of 18°C to 10 or 5°C, than under long days (LD 16:8). This mimics the situation in northern Japan (Sapporo, Hokkaido) where natural populations of *D. auraria* enter diapause and become cold tolerant in October as the mean daily temperature falls below about 15°C and day length below 12 hours.

Maternal short days induce larval diapause in the blow fly, *Calliphora vicina* (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a). Saunders and Haywood (1998) showed that survival to eclosion, after exposure to up to 18 days at -8°C, was greater for diapausing larvae of *C. vicina* than for same-age, nondiapausing, pupae. Survival was also greater for diapause-destined than nondiapauses-destined larvae from two northern strains (Finland, 65°N; Scotland, 55°N), but not for a strain isolated from an area further south (Italy, 44°N). These results indicate that diapause-related cold tolerance may also have a geographical dimension, with a latitudinal cline alongside that for critical day length (see Chapter 10) and diapause intensity.

D. SEASONAL MORPHS

Season-bound morphological forms have been described in the Lepidoptera, Orthoptera, Homoptera, Heteroptera and in the Thysanoptera. In most of these instances photoperiod is the decisive environmental factor involved. At certain points in development, day length appears to operate a genetic switch thereby opening or closing alternate pathways of development. The long days of summer, for example, may favour the production of darkly pigmented summer forms of butterflies, short-winged bugs, or viviparous parthenogenetic aphids that reproduce rapidly to take advantage of favourable food supplies. Short days, on the other hand, often favour winged sexual forms that become dormant or, in the case of aphids, lay diapausing eggs. The control of seasonal polymorphism (polyphenism) is therefore frequently associated with the regulation of diapause or nondiapauses development.

Lepidoptera. The nymphalid butterfly *Araschnia levana* provides one of the best-known examples of seasonal dimorphism in the Lepidoptera. This species exists in two forms - *levana* and *prorsa* - which at one time were regarded as distinct species. Danilevskii (1948) and Müller (1955), however, showed that the short days of autumn induced a diapausing pupa from which the 'typical' *levana* form (orange-brown with a pattern of black spots) emerged in the spring. The long days of summer, on the other hand, led to the production of the black and white *prorsa* that was not associated with a pupal diapause. In the southern part of Russia the green silver-lined moth *Hylophila prasinana* also occurs in a spring and summer form. The spring form, which is the typical *prasinana*, emerges from a diapause pupa, whereas the summer form (formerly thought to be a distinct species, '*H. hongarica*') does not include a diapause stage in its life cycle. Further north this species is univoltine and only the *prasinana* form is known (Danilevskii, 1965). Sakai and Masaki (1965) have similarly shown that short photoperiods (less than 13 hours) induced the spring and autumn form of *Lycaena phlaeas daimio*, which shows a ground colour of bright coppery-orange with small black spots and a narrow marginal band. On the other hand, long photoperiods (more than 14 hours) induced the more darkly pigmented summer form. This species also possessed a photoperiodically determined diapause (in the larva) but the induction of diapause appeared to be independent of wing coloration. Most larvae at high temperature, for example, even in short day length, averted diapause, but still gave rise to the appropriate wing colour. Similar seasonal effects are known in the great southern white butterfly, *Ascia monuste* (Pease, 1962); this species produced a melanistic summer form in LD 16:8 but a white form in short days (LD 8:16). Further examples are afforded by the nymphalids *Polygonia c-aureum* (Aida and Sakagami, 1962) and *Kaniska canace nojaponicum* (Aida, 1963), and in the pierids *Eurema hecabe mandarina* (Aida, 1963) and *Colias eurytheme* (Watt, 1969; Hoffmann, 1973, 1974). Tsurumaki et al. (1999) more recently showed that a bivoltine race of the commercial silk moth, *Bombyx mori*, also showed a seasonal diphenism. Larvae developing under the long days of summer, and high temperature, became autumnal diapause egg producers with a greater number of dark brown scales on the wings. On the other hand, larvae developing under the short days of spring, and lower temperature, became summer nondiapause egg producers with comparatively light coloured wings.

Several species of *Pieris* in western North America show seasonal morphs. Populations of *P. napi* from coastal California, for example, are bivoltine and occur in two phenotypes, a spring *venosa* form with heavy black scaling on the veins of the hind wings, and a lighter summer form (*castoria*) almost devoid of black scales. Inland populations, on the other hand, are univoltine and monophenic, all adults being of the *venosa* type. Both stocks are capable of producing both phenotypes, however, the dark spring form emerging from over-wintering diapause pupae, and the lighter summer form from nondiapause pupae (Shapiro, 1975a, 1977). *Pieris occidentalis* also occurs in two phenotypes, dark and light, the former (*calyce*) developing from larvae reared in short day length (LD 6:18 and 10:14) and the latter from larvae in long days (>LD 14:10) (Shapiro, 1973). Far-northern populations of this species (from Alaska) are all univoltine and of the dark vernal type, but still retain the ability to produce the light aestival type from nondiapausing pupae when reared at high temperature and very long photoperiods (Shapiro, 1975b).

In most cases the selective advantages of these seasonal colour polymorphisms in Lepidoptera are far from clearly understood. In *Colias eurytheme*, however, Watt (1968, 1969) has shown that the dark hind undersides of the spring and autumn (short day) broods increased the efficiency of the absorption of solar energy, thus promoting activity and reproductive

success during the cooler periods of the year. The light yellow or orange hind under-wings of the summer generation, on the other hand, minimised overheating in the warm season (see also Hoffmann, 1978). It is not known how widespread this phenomenon is, but in the case of *Araschnia levana*, *Lycaena phlaeas* and *Ascia monuste*, long-day summer forms are darker than the short-day broods of spring and autumn.

The vapourer moth, *Orgyia thyellina*, shows seasonal changes in wing length, females emerging in summer having normal wings, but those in the autumn being brachypterous (Kimura and Masaki, 1977). Summer macropteres lay nondiapausing eggs, whereas the autumnal brachypterous lay diapausing eggs that are also larger in size and darker (Fig.9.7). All of these seasonal changes in morphology are controlled by photoperiod: brachypterous are produced from larvae reared at short day length (LD 12:12 to LD 14: 10) and long-winged forms at photoperiods longer than the critical (between LD 15:9 and 16:8).

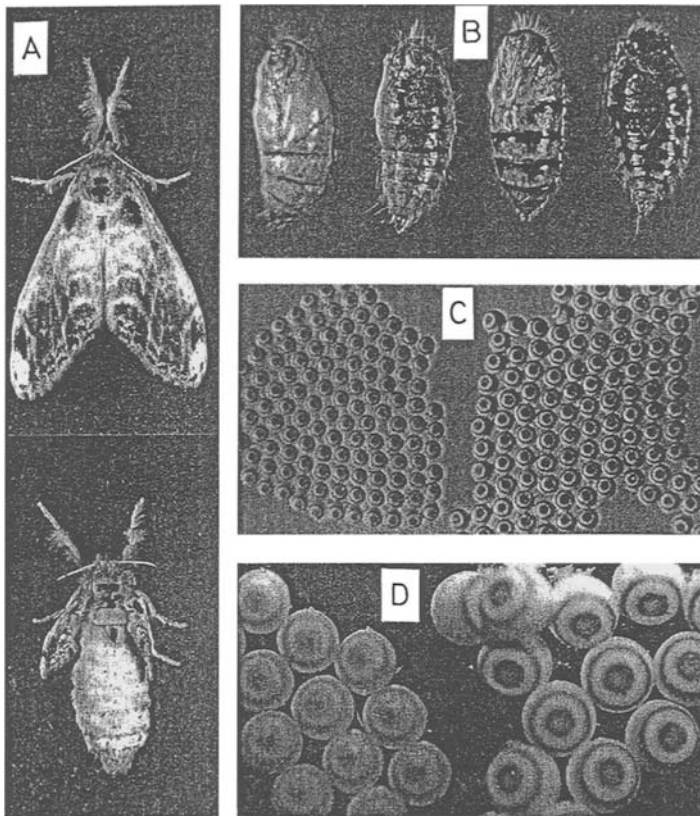


Fig. 9.7. Photoperiodically induced variation in *Orgyia thyellina*. A – macropterous (long day) and brachypterous (short day) females; B – pale (long day) and dark (long day) pupae; C – diapause (short day, right) and nondiapauses (long day) eggs; D – ditto under higher magnification. From Kimura and Masaki (1977).

Hormonal regulation of seasonal morph determination has been investigated in a number of Lepidoptera. In *Papilio xuthus*, spring and summer forms are determined, apparently coincidentally, with diapause and nondiapauses development, by larval exposure to short and

long days respectively. In this species, experiments involving parabiosis and brain transplantation between diapause and nondiapause pupae (Endo and Funatsu, 1985) indicated that the brain of the latter secreted a humoral factor producing the summer morph. A similar result was obtained for the small copper butterfly, *Lycaena phlaeas daimio* (Endo and Kanata, 1985). The humoral factor was identified as a *summer morph producing hormone* (SMPH) which caused the wings of the summer form to become brownish, rather than the bright coppery red of the spring generation. Since application of ecdysteroids to newly formed pupae made wing colour redder, a role for ecdysteroids was also proposed.

In elegant experiments using the nymphalid *Araschnia levana*, Koch and Bückmann (1987) constructed parabiatic pairs between short-day, diapausing, pupae and long-day, nondiapausing pupae. Both subsequently underwent nondiapause development, but the type of adult produced was dependent on the time of joining. If parabiosis was performed one day after pupation the resulting adult was of the black and white summer form; if it was performed four days after pupation, the reddish spring morph developed. Removal of the pupal head and prothorax prevented adult development. Subsequent injection of 20-hydroxyecdysone (20-HE) into the remaining part of the body, however, produced summer morphs with three-day old pupae, but spring morphs with ten-day old pupae. These results indicated that seasonal diphenism in *A. levana* was regulated by the *timing* of the ecdysteroid release initiating adult development.

In another nymphalid, *Precis coenia*, Rountree and Nijhout (1995) showed that removal of the brain caused all animals to develop into the summer *rosa* form, regardless of environmental conditions. The autumnal *linea* phenotype, however, could be induced in brainless pupae by timed injections of 20-hydroxyecdysone (20-HE). The critical period for sensitivity to 20-HE was between 28 and 48 hours after pupation. Measurements of ecdysteroids revealed that titres in long-day pupae, destined for development into the light beige *linea* form, began to rise about 20 hours after pupation. Under short days, however, titres were delayed until about 60 hours after pupation, giving rise to the reddish-brown *rosa* form. The regulation of seasonal morphs in both *Araschnia levana* and *Precis coenia* was therefore considered to be the result of the timing of ecdysteroid release. There was no need to postulate the existence of a summer-morph producing brain factor.

Orthoptera, Thysanoptera and Heteroptera. Seasonal forms in the Orthoptera, Heteroptera and Thysanoptera frequently include alary dimorphism. In the ground cricket *Nemobius yezoensis*, for instance, experimental exposure to long days induced some of the nymphs to develop without a diapause and to become long-winged macropteres (Masaki and Oyama, 1963). Normal development in this species, however, was univoltine, and the insects were short winged, diapausing as a nymph. The band-legged ground crickets, *Pteronemobius fascipes* (Masaki, 1973) and *P. taprobanensis* (Tanaka, 1976; Tanaka et al., 1976), have a similar photoperiodically determined wing dimorphism. Long-winged forms of *P. taprobanensis* were obtained in large numbers, for example, by transferring nymphs from short to long day lengths during their development.

Working in southern Finland, Vepsäläinen (1971 a, b) found that the heteropteran *Gerris odontogaster* occurred in short- and long-winged morphs. The over-wintering population was almost entirely macropterous. Their offspring, however, were dimorphic, micropteres emerging up to the middle of July and macropteres thereafter. These long-winged individuals left the pond for over-wintering sites and hibernated in a state of reproductive diapause. Experimental manipulation of the photoperiod showed that long day length (over 18 hours per day), plus incremental changes in day length during the early nymphal instars, was required to produce

adult micropteres. In the Thysanoptera, Köppä (1970) demonstrated that micropterous specimens of *Anaphothrips obscurus* were produced by short-day illumination.

Another example concerns the stink bug, *Euschistus tritium*. McPherson (1974) showed that nymphs reared in LL produced adults with spinose shoulders and zero to two abdominal mid-ventral spots, characters of the summer morph, '*E. tritium pyrrhocerus*'. In contrast, those reared in short days (LD 12:12) gave rise to adults with sub-triangular shoulders and three to four spots, typical of the vernal and autumnal morph, '*E. tristigmus tristigmus*'. *Thyanta calceata*, another pentatomid, also showed two seasonal phenotypes, a summer form with green integument and a shorter pubescence and a spring and autumn form with a brown integument and a long pubescence. McPherson (1978) showed that the brown adults were produced from nymphs raised in short day lengths (LD 8:16 to LD 12:12) whereas the green aestival form was produced when photoperiod exceeded the critical value (about 12½ hours per day).

Homoptera. It is probably among the Homoptera that seasonal polyphenism is most widespread, particularly in the leaf hoppers (Cicadellidae), white flies (Aleyrodidae), jumping plant lice (Psyllidae) and, particularly, in the aphids (Aphididae). Müller (1954, 1957), for example, showed that the leaf hoppers *Euscelis plebejus* and *E. lineolatus* each occurred in a number of seasonal forms, many of which had previously been given separate specific status (Fig. 9.8). These forms differed in a number of characters including size, colour and the shape of the male genitalia. *E. plebejus* was found to be sensitive to day length in the middle nymphal instars. In short-day conditions (4 to 15 hours light per day) only the spring form (*incisus*) was obtained, but in long days (17 to 18 hours) nearly all became the summer or *plebejus* form. At intermediate and ultra-short day lengths, or in DD, leafhoppers with an intermediate type of genitalia were produced. The cicadellids *Nephotettix apicalis* and *N. cincticeps* (Kisimoto, 1959), the delphacids *Stenocranus minutus* (Müller, 1954) and *Delphacodes striatella* (Kisimoto, 1956), and the psyllid *Psylla pyri* (Bonnemaïson and Missonnier, 1955; Oldfield, 1970) all showed photoperiodic control of size, wing dimorphism and diapause. The whitefly *Aleurochiton companatus* produced non-diapause and unpigmented 'puparia' during long days, but diapausing 'puparia' with thick sclerotised cuticles during the autumn (Müller, 1962, a, b).

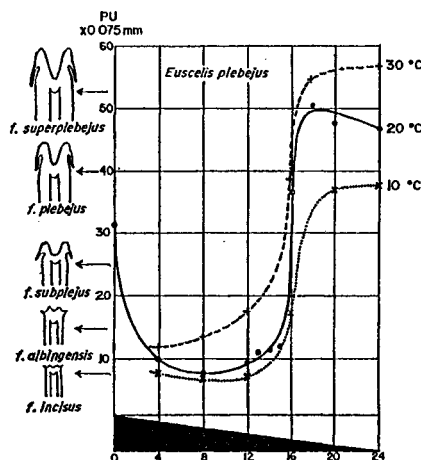


Fig. 9.8. Photoperiodic control of polymorphism in *Euscelis plebejus*. The aedeagus in the various forms is shown on the left; the ordinate is based on measurements of the aedeagus outline. After Müller (1960).

The most complex seasonal cycles are undoubtedly found in the aphids. Most temperate species reproduce during the summer months as a series of viviparous, parthenogenetic females usually referred to as *virginoparae* - which may be alate (*alatae*) or wingless (*apterae*) - but produce sexual forms (males and *oviparae*) as the days become shorter in the autumn (Fig. 9.9). The fertilised *oviparae* generally deposit diapausing eggs that over-winter on the primary host. This alternation of sexual and asexual forms is referred to as *holocycly*. The rapid multiplication, viviparity and parthenogenetic reproduction of the *virginoparae* allow maximum utilisation of available resources during the summer months, whereas sexual reproduction allows genetic recombination and the production of the hibernating stage.

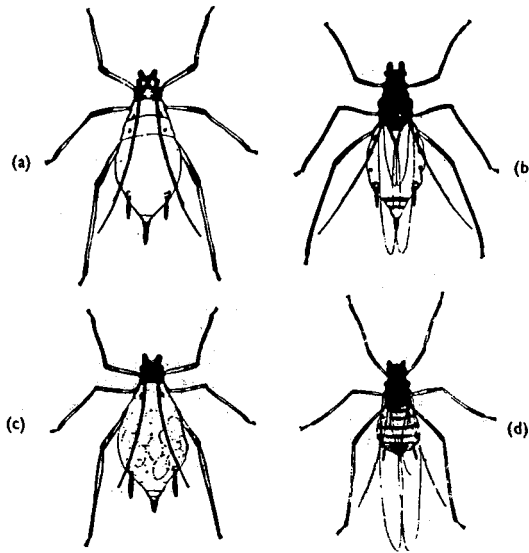


Fig. 9.9. Seasonal and sexual forms in the aphid *Megoura viciae*. A - apterous virginopara formed under long days; b - alate virginopara formed under long days and crowded conditions; c - ovipara formed under short days; d - male. After Lees (1959).

Some aphids such as *Aphis forbesi* (Marcovitch, 1924), *Brevicoryne brassicae* (Bonnemaïson, 1951) and *Megoura viciae* (Lees, 1959) are *monoecious*, spending their active life on a single plant species. Others such as *Aphis fabae* (Davidson, 1929), *Myzus persicae* (Bonnemaïson, 1951) and *Dysaphis plantaginea* (Bonnemaïson, 1958, 1965) are *heteroecious* and change their host plant during the season, often from a primary host (a tree, for example) to a variety of herbaceous plants during the summer months. In different aphid species the determination of sex, and the determination of virginoparae and oviparae, are under photoperiodic control; the production of alate forms, however, is frequently dependent on population density. In *Myzus persicae* (Blackman, 1975), *Acyrtosiphon pisum* (Sharma et al., 1975) and *Aphis fabae* (Tsitsipis and Mittler, 1977a), males are produced in response to short days, in the latter only at low temperature (Tsitsipis and Mittler, 1977b). The production of sexual forms (males and oviparae), however, is often prevented during the short days of spring by a long-range 'interval timer' which over-rides the photoperiodic clock. Such a mechanism

has been described in *Megoura viciae* (Lees, 1960a), the sycamore aphid *Drepanosiphum platanoideus* (Dixon, 1971) and the lime aphid *Eucallipterus tiliae* (Dixon, 1972) (see also Chapter 10, D). Lees (1966) has given a detailed account of aphid morphs and their induction.

More recent studies on the photoperiodic control of wing development in the black bean aphid, *Aphis fabae* (Hardie, 1987b) and of sex and female morph determination in the pea aphid, *Acyrtosiphon pisum* (Lees, 1989, 1990) will be considered in Chapter 10.

The hormonal regulation of female morph determination in aphids appears to involve juvenile hormones (JH) (see reviews by Lees, 1983; Hardie, 1984). Although some early data were controversial, later studies showed that topical application of natural JHs, or their synthetic analogues, could mimic the effects of long days under short day conditions. Conversely, anti-JH compounds such as precocene were capable of mimicking short days under a long day environment. In *M. viciae* and *A. fabae*, the natural JH recovered from whole body extracts by gas chromatography-mass spectroscopy was found to be JH III (Hardie et al., 1985). Although titres were variable and over-lapping, they were higher in *M. viciae* under long days than under short days. In experiments on *A. fabae*, Hardie (1981a, 1981b) showed that JH III was more effective than JH II, and JH I the least. Topical applications early in development promoted apterisation, whereas by the middle instars it promoted maximum juvenilisation. Working with *M. viciae*, Hardie (1987a) showed that corpus allatum volume increased with age under short days but decreased under long days. In long-day insects, cauterisation of the anterior protocerebrum, close to the group I neurosecretory cells considered essential for the production of virginoparae (viviparae) under long days (Steel and Lees, 1977), resulted in a change of progeny type from vivipara to ovipara and an increase in CA volume. Cautery under short days had no such effect. These results suggested that the group I NSC release a neurohormone under long days which stimulates JH synthesis by the corpus allatum (see Chapter 14).

E. GROWTH RATES

In a number of insects the rate of development is controlled by photoperiod and, as with other such phenomena, long- and short-day responses may be observed. Amongst the cutworms, for example, larvae of *Agrotis occulta* developed more rapidly under long days, whereas those of *A. triangulum* developed more rapidly under short (Danilevskii, 1965). Vinogradova (1967) showed that larvae of the mosquito *Aedes triseriatus* developed more rapidly in LD 20:4 than in LD 10:14, the latter causing a pronounced delay during the fourth instar. The sod webworm, *Crambus tutilus*, however, was a short-day species and developed more rapidly in LD 12:12 than in LD 16:8. In addition, larvae of this species exposed to day-lengths decreasing at a rate of 2 minutes per day from an initial 16-hour photoperiod grew more quickly than those exposed to LD 16:8 throughout (Kamm, 1972).

Atwal (1955) showed that larvae of the diamond back moth *Plutella maculipennis* completed their development in about 18 days at LD 9:15 but took only about 15.5 days at LD 15:9. Furthermore, adult females of this species raised in LD 15:9 laid more eggs than those raised in LD 9:15, even though those reared at the shorter day length had developed more slowly and might be expected to be heavier and to give rise to more fecund adults. This effect of photoperiod on fecundity was confirmed by Harcourt and Cass (1966) who showed that females of *P. maculipennis* raised from the larval stage in LD 12:12 laid about 37 eggs whereas those raised in LD 16:8 laid about 74. According to these authors there is no 'true' diapause in *P. maculipennis* although the species over winters most frequently as an adult, and short-day females deposited more fat and developed fewer eggs than those raised in long days. This

species, therefore, may possess a weak or incipient form of ovarian diapause. Short-day promotion of ovarian development is indicated in the results of Meudec (1966). These showed that the ovaries of 24-hour-old females of *Acrolepia assectella* contained more eggs when the larvae had been raised in LD 9:15 (8.3 eggs per female) than when raised in DD (4.1 eggs per female) or in LL (2.9 per female). Minis and Pittendrigh (1968) showed that the embryonic development of *Pectinophora gossypiella* was more rapid in LL than in DD. This so-called 'light-growth effect' is probably another example of the same phenomenon. It also seems clear that these photoperiodic effects on growth and development are similar to those effects of environmental periodicity on growth and survival discussed in Chapter 6. In the flesh-fly *Sarcophaga argyrostoma*, for example, larval growth rates were affected by photoperiod and the length of the driving light cycle (see Fig. 6.19) (Saunders, 1972).

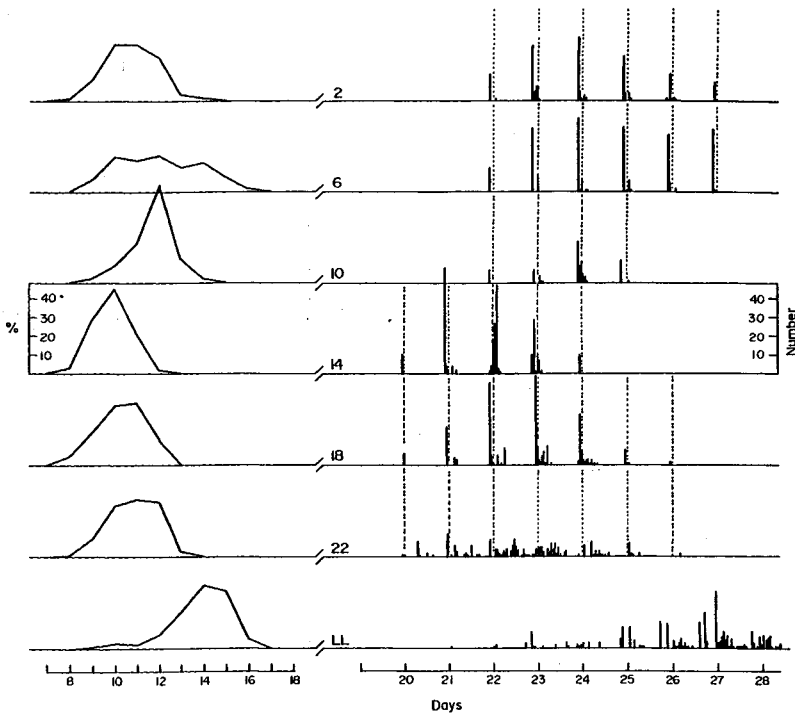


Fig. 9.10. Photoperiodic control of developmental rate in *Sarcophaga argyrostoma*. Cultures maintained under 2, 6, 10, 14, 18 and 22 hours light/day, and in LL. Polygons on the left show the per cent puparium formation per day in each culture. Vertical black bars on the right show the hourly distributions of pupal eclosion. Dotted vertical lines record the times of the light-on signal ('dawn') in each regime. Developmental time is shortest in long days (LD 14:10, LD 18:6) and longest in short days (LD 6:18, LD 10:14) and in LL. Eclosion is rhythmic in all regimes up to LD 18:6, thereafter it becomes arrhythmic (see Chapter 3). From Saunders (1976).

In many instances photoperiodic effects on larval growth rate appear to be associated in some way with the diapause response. In the viceroy butterfly *Limnitis archippus*, for example, larvae maintained in long days, showed rapid development to the adult instar without any arrest. In short-day conditions, however, larval development was much slower and the

larvae spun silken hibernacula in the third instar within which they became dormant (Clark and Platt, 1969). Similarly, in the flesh flies (*Sarcophaga* spp.), short days induced slow development, a protracted post-feeding wandering period, and subsequent pupal diapause whereas long days induced rapid development and non-diapausing pupae (Denlinger, 1972; Saunders, 1972). The longer wandering period might be of selective advantage in allowing greater time for the diapause-destined larva to find a suitable overwintering site. Protracted larval development, however, is also one of the variables that raises the incidence of pupal diapause because it allows a greater number of inductive short-day cycles to be seen by the larvae before the end of the sensitive period (see Chapter 12). Day length may also act upon developmental time independently from diapause induction (Saunders, 1976). For example, cultures of larvae deposited by females kept at high temperature (25°C) under continuous light were maintained in a variety of short- and long-day regimes (Saunders, 1976) (Fig. 9.10). Despite the fact that the high larval incubation temperature (25°C), coupled with the embryonic experience of constant light, prevented the appearance of diapause in the pupae, larval development was slower under short days (LD 2:22, 6:18 and 10:14) than long days (LD 14:10, 18:6 and 22:2). Development was also protracted in the aperiodic regime of continuous light. The hypothesis that diapause incidence is a result of a complex interaction between the diapause inducing or averting effects of photoperiod and the number of such cycles experienced during the 'sensitive period' will be examined further in Chapter 12.

Beck (1986, 1988) showed that a daily *thermoperiod* (see also Chapter 10, A4) stimulated larval growth rate in the cutworm *Agrotis ipsilon*, a species apparently without a diapause in its life cycle. Growth rate, assessed by weight gain and head width, was found to be greatest in DD and a thermoperiod (12 hours at 20°, 12 hours at 10°), less in LD 12:12 with a concomitant temperature cycle, and least in LD 12:12 at a constant temperature of 15°C.

Working with a Czech population of the linden bug, *Pyrrhocoris apterus*, Saunders (1983) showed that the rate of nymphal development was significantly slowed under photoperiods close to the critical day length for the induction of adult (ovarian) diapause. It was suggested that retardation of development under these near-critical photoperiods might allow an increased time for the accumulation of important inductive photocycles, thereby 'fine-tuning' the diapause response. This phenomenon was later confirmed by Numata et al. (1993) in a Russian population of *P. apterus*. Rather similar data were obtained for the ground cricket, *Dianemobius nigrofasciatus* (Kidokoro and Masaki, 1978), who also considered the phenomenon to be a 'fine-tuning' mechanism.

F. MIGRATION

The role of photoperiod in the control of insect migration has received relatively little attention despite the fact that a large number of species display seasonal movements, often over quite long distances. Southwood (1962) considered that migration and diapause were two alternatives open to insects in order to counteract changes in their environment. Insects may enter diapause *in situ*, or move to more favourable areas. Other insects, such as the pentatomid *Eurygaster integriceps*, the pond skater *Gerris odontogaster*, the monarch butterfly *Danaus plexippus* and various coccinellid beetles, to name but a few, migrate to hibernation sites and return to breeding and feeding areas in the spring. Aphids also migrate from summer to winter hosts. It is clear that many of these migrations are closely associated with the induction of the diapause syndrome, and in many cases are controlled by day length. Dingle (1978) and Tauber et al. (1986) have reviewed the photoperiodic inputs into insect migration strategies; these will not be covered in detail here.

The classical example of a long-distance migrant is the monarch butterfly, *Danaus plexippus* (Brower, 1977). During the summer months this species occurs throughout most of the United States and southern Canada where it produces several nondiapauses generations on milkweed (*Asclepias* spp.). Every autumn, large numbers of monarch butterflies fly south to hibernation sites. Western populations fly down the Pacific coast to congregate in overwintering sites on just a few trees – for example, on the Monterey peninsula, south of San Francisco. Eastern and mid-western populations use a different route to localities in Mexico, Guatemala and Honduras where they congregate in vast assemblages of up to several million individuals. The southerly migrations are carried out by insects in an ovarian diapause characterised by a cessation of ovarian development and fat body hypertrophy. Diapause is maintained in the overwintering sites by short day length and cool temperatures. Termination of diapause occurs with the onset of long days and increased warmth (and treatment with juvenile hormone). Mating occurs in February and the mated butterflies then begin migration back to northerly breeding sites.

The coccinellid beetles, *Semiadalia undecimnotata* and *Coccinella septempunctata* were shown to enter a reproductive diapause under short day length (LD 8:16 and LD 12:12) but not under long day length (LD 16:8 and LD 19:5) (Hodek and Cerkasov, 1960; Hodek, 1967). Once diapause had been induced both males and females migrated to winter hibernation sites, the females with undeveloped ovaries and large reserves of fat. In the Czech Republic, *C. septempunctata* moved to winter refuges amongst cultivated areas such as pastures and the edge of woods, often in elevated areas. *S. undecimnotata* migrated to hibernation sites on or near the summits of hills, and overwintered in large aggregations in rock crevices or at the base of plants (Hodek, 1960). In the spring the reactivated insects dispersed in any direction.

The migration strategies of the milkweed bug *Oncopeltus fasciatus* have been studied by Dingle (1972, 1974) and Caldwell (1974). This species occurs from Brazil up to Canada. In the spring it migrates northwards up the Mississippi valley, aided by southerly winds, colonising temporary stands of milkweed (*Asclepias* spp.); in the autumn it migrates southwards along the same route after producing two to four summer generations. It is unable to overwinter in the northernmost parts of its distribution. As with many other insect migrants, migration occurs in young post-teneral adults before the start of reproduction, and the migrating insects show many features characteristic of the diapause syndrome, such as suppressed ovarian development and fat body hypertrophy. During migration itself, vegetative functions, such as feeding, mating and reproduction, are suppressed in favour of locomotion that is sustained and unidirectional.

The propensity to migrate in *O. fasciatus* is genetically 'programmed', but is also under environmental control. For example, whilst oviposition began after 13 to 15 days in bugs raised under long days (LD 16:8, at 23 °C), it was delayed for up to 80 days or more under short days (LD 12:12) (Dingle, Brown and Hegmann, 1977). Short days thus induced a reproductive diapause that provided a greater time for the southerly autumnal migration. Short days also appeared to increase the proportion of bugs undergoing prolonged migratory flights, and other factors such as starvation, crowding and perhaps the lack of mating partners, also delayed oviposition and therefore contributed to the time available for migration. Northerly migrations in the spring when days are lengthening might be maintained by the lack of milkweed seed that is necessary for oviposition.

The idea that migration or overwintering *in situ* are alternate strategies is nicely exemplified by a comparison between two species of milkweed bug in North America: *Oncopeltus fasciatus* and *Lygaeus kalmii* (Caldwell, 1974). Whilst the former is a long distance migrant incapable of overwintering in the northerly parts of its range, *L. kalmii* enters an adult

reproductive diapause in hibernacula, over winters throughout its range, and makes only short-distance flights to utilise temporary stands of milkweed.

G. MISCELLANEOUS PHOTOPERIODIC PHENOMENA

This section includes an assortment of seasonal phenomena regulated by day length. Some of these are components of the diapause 'syndrome' (reduced metabolism, accumulation of metabolites, acquisition of cold tolerance, and changes in behaviour) already mentioned; others may be independent of the diapause condition.

Pittendrigh (1961) showed that recovery of adults of *Drosophila melanogaster* from heat stress (40°C for 12 minutes) was more rapid under long days (e.g. LD 18:6) than under short days (e.g. LD 6:18). In the house fly *Musca domestica*, which is considered to be 'day-neutral' (Danilevskii, 1965), adults raised as larvae in LD 14:10 were more susceptible to DDT, dieldrin and aldrin than those raised in LD 10:14 or in LL (Fernandez and Randolph, 1966). These two examples show that photoperiod may exert effects on quite fundamental aspects of physiology.

Working with a north Italian strain of *Locusta migratoria*, Perez et al. (1971) demonstrated that male sexual behaviour was a function of day length. Males were raised either in short day length (LD 12:12) or in long day length (LD 16:8). Those males raised in short days spent about 70 per cent of their time in vigorous sexual behaviour for the first 5 or 6 weeks of their adult life. On the other hand, those males raised under long days exhibited only slight sexual behaviour, or none, during the first 2 or 3 weeks, and only reached their maximum (about 60 per cent of their time) after about 10 weeks. In this species there is no clear-cut diapause, although females maintained in short days produced many more egg pods, and more rapidly, than those kept in long days.

Photoperiodic control of mating behaviour was also described in the long-day species *Oncopeltus fasciatus* (Walker, 1978). Under short days, mating activity was reduced to 15 to 20 per cent of normal, photoperiod acting solely on the male. The type of calling song in males of the tettigoniid *Neoconocephalus triops* was also a product of the photoperiodic regime during development (Whitesell and Walker, 1978). In northern Florida this species occurs in two seasonal populations, each with a different wing-stroke rate and calling pattern. Those that were reared in short photoperiod (LD 11:13) entered a reproductive diapause with 'winter' males calling in January to April with an 'uninterrupted' pattern of about 90 wing strokes per second. Those raised in long days (LD 15:9), however, gave rise to non-diapausing 'summer' males calling in July and August with a characteristic pattern of 'interrupted' song with a wing-beat frequency of about 110 beats per second. Other effects of photoperiod on behaviour are seen in adults of the mosquito *Culex pipiens* which did not host-seek or blood feed while in diapause because of a lack of antennal olfactory receptors for the host's lactic acid (Bowen et al., 1988; Bowen, 1990).

Lumme et al. (1972) demonstrated a complex effect of photoperiod on the testis pterin content of a non-diapausing strain of *Drosophila littoralis*. Both long days (LD 18:6) and very short days (LD 6:18) caused a marked decrease in pterin content as compared with either LL, LD 12:12 or DD. This work is of interest because the amount of pterin in the testes is an indication of the amount of that pigment in the whole insect, and pterins have an absorption which coincides with the action spectrum for the phase-setting of circadian oscillations (Chapter 3).

In the ichneumon *Compoletus perdistinctus* the sex ratio was reported to be affected by photoperiod, the greatest proportion of females being produced at LD 12:12 (Hoelscher and Vinson, 1971). Suzuki (1981) also noted an effect of photoperiod on the production of male eggs by foundresses of the wasp *Polistes chinensis antennalis*. In the damselflies *Enallagma hageni* and *E. aspersa*, abnormal wing-pad development occurred at higher temperature and under short days (Ingram, 1976), and Wardhaugh (1977) reported an effect of photoperiod on the shape of the egg pods produced by the grasshopper *Chortoicetes terminifera*.

In many cases photoperiodic phenomena regulate a switch in metabolism as the insects enter diapause: adult insects entering a winter or summer diapause, for example, may lay down fat instead of developing their ovaries. One such example is the mosquito *Culex tarsalis*, which enlarges its fat body under the influence of short days as part of its preparation for winter diapause (Harwood and Halfhill, 1964). Harwood and Takata (1965) demonstrated that overwintering females of *Culex tarsalis* (i.e. those raised under short day length) laid down a greater proportion of their fat in the form of unsaturated fatty acids. In southerly populations of *Culiseta inornata*, however, reproductive dormancy supervened in the hot summer months and females kept at long day length (LD 16:8) showed fat body hypertrophy but a decrease in blood feeding rates (Barnard and Mulla, 1977).

Ovarian diapause in the Colorado potato beetle *Leptinotarsa decemlineata* was induced by short days (LD 10:14) and was associated with the synthesis of 'diapause proteins'; some of these were stored in the fat body. Under long days (LD 18:6), on the other hand, specific vitellogenins were produced which accumulated in the developing oocytes (Dortland, 1978). Other important examples of diapause-associated storage proteins include the beetle *Dendroides canadensis* (Howarth and Duman, 1983), the southwestern corn borer, *Diatraea grandiosella* (Chippendale, 1984) and the wood cockroach *Parcoblatta pensylvanicus* (Wassmer and Page, 1993; Wassmer et al., 1996).

MacLeod (1967) showed that the assumption of the winter colour (pale green, yellow or brown) in adults of the lacewing *Chrysopa carnea* was associated with a reproductive diapause brought about by exposure to short days. Long days induced full summer coloration (bright green) and no diapause. Tauber et al. (1970) further demonstrated that full winter coloration (waxy green or brown with reddish-brown markings on the dorsum), and a more intense diapause, were brought about by a transfer from long days in the immature stages to short days as adults. Effects of such changes in photoperiod will be reviewed in Chapter 10.

ANNOTATED SUMMARY

1. In classical photoperiodism insects are able to distinguish the long days (or short nights) of summer from the short days (or long nights) of autumn and winter, and respond with seasonally appropriate switches in metabolism. Some species are 'active' during long days; others are 'active' during short days. Photoperiodic switches control diapause induction, diapause termination, seasonal morphs (polyphenism), growth rates, migration strategies and a variety of associated physiological states.
2. Diapause involves the temporary inactivation or alteration of the endocrine system, triggered by an appropriate photoperiodic stimulus acting on the brain. Larval-pupal diapause generally involves an inactivation of the brain-prothoracic gland system with a resulting low titre of ecdysteroids; adult, or reproductive, diapause occurs as an inactivation of the brain-corpus allatum system, a lack of juvenile hormone (JH), and gonotrophic dissociation. Some larval diapauses (in the Lepidoptera) are controlled by particular titres

of JH, whilst some embryonic ('egg') diapause is imposed on the embryo by a maternally produced 'diapause hormone' (DH).

3. Insects at higher latitudes may enter diapause (hibernation) as winter approaches in response to short days (long nights) acting as a 'token stimulus'. In areas with a hot, dry summer, insects may enter a summer (aestivation) diapause in response to long days. Within the tropics insects may also enter diapause, often during a dry season, although day length might not be the environmental trigger.
4. Entry into diapause is usually associated with a number of physiological and behavioural characteristics such as reduced metabolism, enhanced stores of fat and proteins, and acquired resistance to cold or heat, which together make up the 'diapause syndrome'.
5. Quiescence differs from diapause in that it is a response to adverse environmental conditions, e.g. desiccation or cold. It does not occur in response to photoperiod, and is quickly lifted with the advent of a more favourable environment.
6. Müller's classification of diapause includes an obligatory *parapause* in univoltine species; *eudiapause* in which a facultative arrest of development is induced by photoperiod but terminated by chilling; and *oligopause*, a facultative arrest induced *and* terminated by photoperiod.
7. The stage at which diapause occurs is nearly always species-specific, but even closely related species may enter diapause in a different instar. Some long-lived insects may diapause in successive winters.
8. Cold tolerance may or may not be associated with diapause. In some species, however, it forms an integral part of the diapause syndrome and may involve the synthesis of 'anti-freeze' proteins.
9. Seasonal polymorphism (polyphenism) occurs in several orders, most notably in the Lepidoptera with summer and winter forms, and in the Homoptera with complex aphid life cycles involving vivipara (virginoparae) in the summer and oviparae appearing in the autumn.
10. Photoperiodically regulated migration may occur in association with diapause and involve either short distance movements to overwintering sites or, as in the case of the Monarch butterfly, long distance migrations from north to south, and back again, between summer breeding grounds and winter hibernation sites.
11. Photoperiod may also influence growth rate, reproductive behaviour, sex ratio and other phenomena, often in relation to diapause onset.

CHAPTER 10

THE PHOTOPERIODIC RESPONSE

As on this whirligig of Time/We circle with the seasons.
Alfred, Lord Tennyson

CONTENTS

Introduction	299
A. <i>Types of Response</i>	300
1. Reactions to stationary photoperiods	300
2. Quantitative responses to photoperiod	304
3. Reactions to sequential changes in photoperiod	304
4. Thermoperiod and photoperiod-thermoperiod interactions	307
B. <i>The Sensitive and Responsive Stages</i>	310
C. <i>Maternal Induction of Diapause and Seasonal Forms</i>	311
D. <i>Factors which Modify the Photoperiodic Response</i>	313
1. Temperature	313
2. Diet	318
3. Density effects	321
E. <i>Geographical Clines in Photoperiodic Traits</i>	321
F. <i>The Genetics of the Photoperiodic Response</i>	327
1. Selection for high and low diapause	328
2. Crossing strains with different photoperiodic and diapause characteristics	328
G. <i>The Spectral Sensitivity and the Intensity Threshold of the Photoperiodic Response</i>	331
Annotated Summary	336

INTRODUCTION

THE range of phenological events controlled by photoperiod was reviewed in Chapter 9. It includes, *inter alia*, the introduction and termination of diapause, the control of seasonal morphs, the regulation of growth rates, and migration. Undoubtedly, each is a complex of widely differing phenomena; diapause, for example, may be hibernial or aestival, may occur at different developmental stages, may be controlled by quite different endocrine mechanisms, may be variously classified as oligopause, eudiapause, photoperiodic quiescence, etc., and may be of different 'strengths', 'intensities' or 'depths'. (For diapause intensity or duration, see Tauber et al., 1986; Saunders, 1987a and Beck, 1989). All of these responses, however, have one thing in common: the principal environmental trigger for their control is night-length (or

day-length) which is used as a 'noise-free' indicator of season. The ways in which different insect species respond to photoperiod are equally variable, and it is these formal responses which define many of the ecological and physiological properties of the photoperiodic 'clock', which is the subject matter of the present chapter.

A. TYPES OF RESPONSE

1. *Reactions to stationary photoperiods*

Most experimental work on the induction of insect diapause has been carried out using stationary photoperiods. Groups of insects are usually exposed to a series of day lengths (all with a 24-hour period, i.e. $T = 24$), and to DD and LL, throughout their development or 'sensitive period', and the proportion of the population entering diapause plotted as a function of day length. The curves obtained are called *photoperiodic response curves* (PPRCs). Since the majority of insects are summer-active, the most frequent type of curve is the long-day response. In this type, insects grow, develop or reproduce under long days but become dormant under short days. This response is particularly common, therefore, in multivoltine species with a facultative winter diapause. Only a few examples of this type will be given here, the choice generally being restricted to those species which have been investigated most intensively (Fig. 10.1).

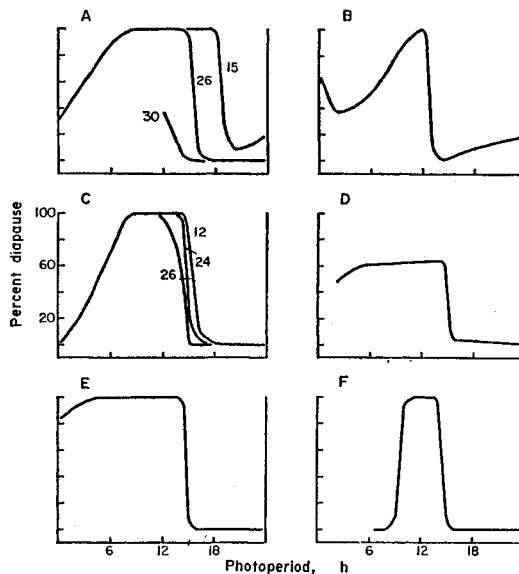


Fig. 10.1. A selection of long-day photoperiodic response curves. A - *Acronycta rumicis* (after Danilevskii, 1965); B - *Pectinophora gossypiella* (after Pittendrigh and Minis, 1971); C - *Pieris brassicae* (after Danilevskii, 1965); D - *Nasonia vitripennis* at 15°C (after Saunders, 1966a); E - *Megoura viciae* at 15°C (after Lees, 1965); F - *Ostrinia nubilalis* at 30°C. (After Beck, 1962.) Figures on the curves indicate temperatures in °C.

The most important feature of the response is the so-called *critical day length* (CDL) that separates the long photophases resulting in nondiapause development from the short

photophases that ultimately lead to the dormant state. The critical day length is frequently very abrupt and, in a sense, is a 'measure' of the accuracy of the clock. In some species, for example, a change of as little as 10 or 15 minutes in the length of the daily light period may result in a significant change in the proportion of the population entering diapause. A change of one hour usually converts all of the individuals from one developmental pathway to the other. The steepness of the curve at the critical point, however, may be a product of selection (accidental or otherwise) occurring during the course of laboratory colonisation and subsequent experimentation. The photoperiodic response curve is also, of course, a 'population response': individual insects presumably have their own threshold, but the response can only be analysed in a group of sufficiently large size.

Danilevskii (1965) pointed out that a photoperiodic response curve includes responses at both natural and unnatural photoperiods. Those towards the right-hand side of the curve, particularly on either side of the critical point, represent those day lengths that occur naturally during that part of the year when the temperature and other climatic factors are suitable for insect development. This part of the curve, therefore, has an adaptive significance and is a product of natural selection (Fig. 10.2). Photoperiods outside this range are never met with in natural conditions or they occur in the depth of winter when insect morphogenesis is at a standstill. Nevertheless, the responses of insects to ultra-short day lengths, and to DD and LL, have a physiological significance; they must be 'explained', for instance, in attempts to determine the mechanism of time measurement (see Chapters 11 and 13).

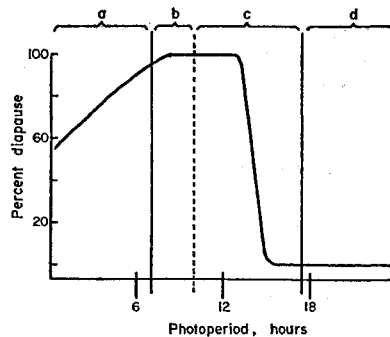


Fig. 10.2. A long-day photoperiodic response curve, showing its properties (schematic). The solid vertical lines indicate the range of natural photoperiods at 55°N. Regions a and d are therefore never experienced in nature. Region b only occurs during the winter when the temperature is probably below the minimum for development (and the insect is in diapause). Only region c is of ecological importance; note that this region is dominated by the critical day length which operates the seasonal switch in metabolism.

Photoperiodic response curves may differ in a number of ways. In some species, such as the Colorado potato beetle *Leptinotarsa decemlineata* (de Wilde, 1958) and the small ermine moth *Yponomeuta vigintipunctatus* (Veerman and Herrebut, 1982), the response in DD is the same (about 100 per cent diapause) as in 'strong' short day lengths. In others, such as the pink bollworm moth *Pectinophora gossypiella* (Pittendrigh and Minis, 1971), the European corn borer *Ostrinia nubilalis* (Beck and Hanec, 1960), the cabbage butterfly *Pieris brassicae* (Danilevskii, 1965) and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1971), the proportion entering diapause declines in ultra-short day lengths. In conditions of constant darkness the response varies from zero as in *P. brassicae* to 100 per cent as in *L. decemlineata*. Diapause

incidence in darkness usually varies widely with temperature. In *P. gossypiella* the proportion entering diapause is apparently greater in DD than in photophases of 2 to 6 hours (Pittendrigh and Minis, 1971). Similarly, under very long photophases and in LL, the incidence of diapause may be higher than in natural long day lengths and, once again, be more variable than at points just longer than the critical (Williams and Adkisson, 1964; Pittendrigh and Minis, 1971). These unstable responses at the extremes presumably reflect the absence of any selective pressure.

In a number of species such as the moths *Leucoma salicis*, *Euproctis similis* (Geispits, 1953) and the beetle *Leptinotarsa decemlineata* (de Wilde, 1958) diapause is induced in all photophases except in a narrow range of long days (Fig. 10.3). These species clearly show a tendency towards a univoltine life cycle that, in an extreme example, would become an obligate diapause in every individual regardless of the photophase. At the opposite extreme, insects such as the house fly *Musca domestica* appear to have no diapause and to be 'day-neutral'. The immature stages of the house fly, for example, pass the winter in a state of quiescence (cold torpor), developing slowly when the conditions allow. The overwintering generation then emerges in the spring and is augmented by migration from the warmer regions (Sacca, 1964; Somme, 1961).

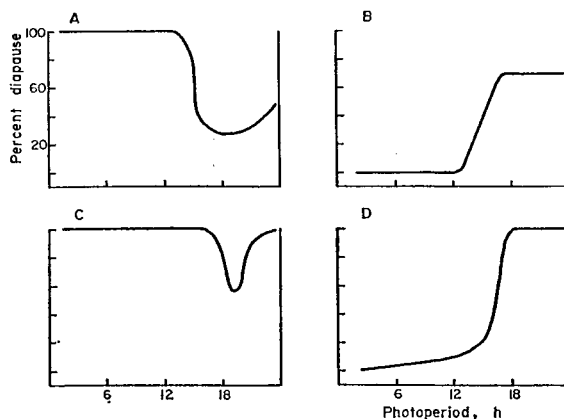


Fig. 10.3. A selection of 'intermediate' (A and C) and short-day (B and D) photoperiodic response curves, A - *Leptinotarsa decemlineata* at 25°C (after de Wilde, 1958); B - *Bombyx mori* at 15°C (after Kogure, 1933); C - *Leucoma salicis* (after Danilevskii, 1965); D - *Stenocranus minutus* (after Müller, 1958).

The opposite, or short-day response, is shown by a smaller number of species, particularly those that are spring-, autumn- or winter-active, and pass the summer in an aestival diapause (Fig. 10.3) (see also Chapter 9). The geometrid *Abraxas miranda*, for example, spends the summer (June to August) in a pupal diapause but the adults emerge in September and October and the larvae of the next generation actively feed and grow during the short days of winter (Masaki, 1957). The commercial silk moth *Bombyx mori*, on the other hand, has a winter diapause in the egg, but a short-day response because the stages sensitive to photoperiod (the eggs and young larvae of the maternal generation) occur during the preceding summer. Thus long days perceived during the summer give rise to moths laying diapausing eggs, whereas short days (i.e. in the spring) give rise to moths laying nondiapausing summer eggs (Kogure, 1933).

A number of species living in southerly latitudes, where the summers are hot and dry but the winters cold, are found to enter diapause in the summer *and* in the winter, and become active at two seasons, spring and autumn. These species may show both long- and short- day responses according to the season. In Japan, southern races of the cabbage moth, *Mamestra brassicae*, for example, have a pupal diapause in the winter and the summer.

Both the hibernal and aestival diapauses are induced by photoperiod, and are of the long- and short-day types respectively (Masaki, 1956, 1968, 1980). In the fall web-worm moth *Hyphantria cunea*, larvae exposed to short days give rise to pupae with a winter diapause (Masaki et al., 1968). Under long-day conditions, however, some pupae undergo a brief summer arrest (Umeya and Masaki, 1969) so that the adults tend to emerge in two distinct peaks. Even in more northerly latitudes long-lived insects may show two dormant periods. Thiele (1969) showed that the beetles *Nebria brevicollis* and *Patrobis atrorufus* hibernate as larvae. The young adults emerging in the spring and early summer then undergo an aestivation diapause before maturation and reproduction.

The pea aphid, *Acyrtosiphon pisum*, may possess two separate photoperiodic clocks showing markedly different response curves (Lees, 1989, 1990). In the late summer, longer scotophases induce the production of males and egg laying females (oviparae). The PPRC for male production, however, was sharply peaked around D = 11 hours, whereas that for ovipara production showed a rise in the incidence of this morph about 1 to 1½ hours later than that for males and then maintained a high plateau until D exceeded 20 hours (Fig. 10.4).

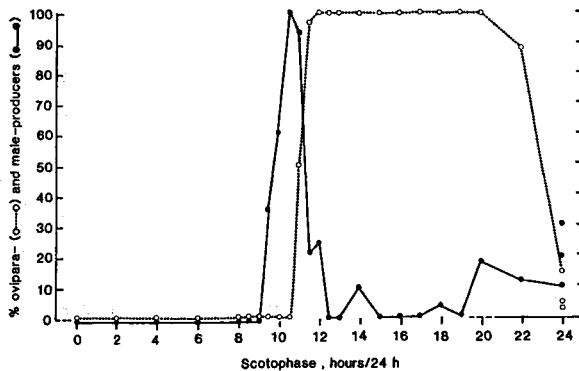


Fig. 10.4. Responses of the pea aphid, *Acyrtosiphon pisum*, to 24 hour photoperiods. Closed circles – male producers; open circles – ovipara producers. (From Lees, 1990).

In the linden bug, *Pyrrhocoris apterus*, the induction and termination of adult (ovarian) diapause showed different critical day lengths (Saunders, 1983). For the strain studied, the critical day length (CDL) for induction was close to 15¼ hours per 24, whereas that for diapause termination was about one hour longer (Fig. 10.5). The termination threshold remained unaltered in unchilled bugs for about 4 to 14 weeks, but the photoperiodic termination of diapause was completely abolished by chilling at 4 °C for 12 to 16 weeks. *P. apterus* appears to be one of the few insects to 'measure' day length rather than night length (Saunders, 1987b).

2. Quantitative responses to photoperiod

In most of the examples reviewed above it has been assumed - for the sake of simplicity - that the photoperiodic clock merely distinguishes long from short days (or more commonly, short from long nights) by a qualitative or all-or-nothing mechanism (see also Chapters 11, 12 and 13). In recent years, however, a number of papers have discussed the possibility of *quantitative* time measurement, i.e. a 'clock' mechanism that distinguishes between *actual* day or night lengths, rather than merely discriminating 'long' from 'short'. These papers include, among others, Tauber and Tauber (1973b), Kimura (1982, 1990), Spieth and Sauer (1991), Gomi and Takeda (1992) and Numata and Kobayashi (1994). The concept of quantitative time measurement was perhaps most fully developed by Zaslavski and Fomenko (1983), and in V.A. Zaslavski's (1988) book, "*Insect Development: Photoperiodic and Temperature Control*".

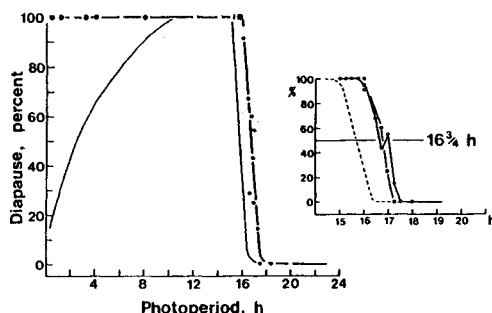


Fig. 10.5. Photoperiodic responses of the linden bug, *Pyrrhocoris apterus*, showing a longer critical daylength for diapause termination than diapause induction (25°C). Open circles - diapause termination by photoperiod after 35 days; closed circles - ditto after 49 days. Also shown as a thin line is the induction curve. Inset: details of the response showing critical daylength for diapause termination (16¼ hours) about one hour longer than that for induction. (From Saunders, 1983).

Several of the more recent models for photoperiodic time measurement (see Chapter 13) incorporate a *quantitative* concept and will be dealt with more fully there. Here it is sufficient to note that even if the 'clock' merely distinguishes 'long' from 'short' nights, a quantitative component could occur at the level of the photoperiodic counter, the mechanism that accumulates successive inductive cycles during the insect's sensitive period (Chapter 12). In that sense all insect photoperiodic clock-counter systems are essentially quantitative. Even under photophases longer than the critical value, diapause may be induced if a *sufficient number* of cycles are accumulated, e.g. in the parasitic wasp, *Nasonia vitripennis*, under LD 18:6 (Saunders, 1966a).

3. Reactions to sequential changes in photoperiod

In the examples discussed above the reactions to stationary photoperiods, either above or below a critical value, were considered. Such conditions, of course, do not exist in nature: natural photoperiods are constantly changing, the rates of change being a function of latitude and time of the year. Except for the summer and winter solstices, each day length occurs twice

in a year, once when the days are increasing and once when they are decreasing. For this reason it is natural that responses to the *direction* of such changes have been sought, particularly amongst those insects which are long-lived. It should be remembered, however, that in many insects the problem of an insect 'deciding' whether days are increasing or decreasing probably does not arise. In some species, for instance, the stages sensitive to photoperiod may only be present at certain seasons, e.g. in the autumn but not in the spring. In others, such as those inhabiting higher latitudes, cold spring weather may delay the resumption of activity well into the season, far beyond the critical day length.

However, some insects do respond to the direction of change in day length by determining a *sequence of photoperiods*, either long to short, or short to long, at different stages of their development. Norris (1959, 1962, 1965), for example, showed that the red locust *Nomadacris septemfasciata* entered an intense reproductive diapause if the hoppers experienced a long-day regime (about 13 hours light per day) but the adults a short day (about 12 hours). On the other hand, diapause-free development followed a transfer from short to long days. In its natural environment (parts of tropical Africa within a few degrees of the equator) this type of response ensures that the species reproduces during the 'summer' rains but becomes dormant during the 'winter' drought. A similar response was reported for the bollworm *Heliothis zea* (Wellso and Adkisson, 1966; Adkisson and Roach, 1971). The most effective treatment for inducing diapause in this species was found to be when the adults and eggs were exposed to longer day lengths than the larvae.

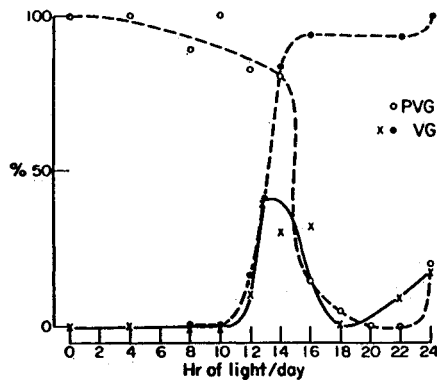


Fig. 10.6. Photoperiodic control of ovarian development in the short-day/long-day beetle, *Pterostichus nigrita*. Open circles - previtellogenesis, occurring in newly emerged females exposed in the autumn to photoperiods of less than 15 hours; closed circles - vitellogenesis, occurring in the spring when photoperiods exceed 13 hours. Beetles normally require a sequence of short days followed by long days for full ovarian maturation, but a proportion can achieve maturation in the stationary photoperiods (13 to 16 hours) enclosed by the solid line (x-x). (From Ferenz, 1977.)

Among carabid beetles, the univoltine spring breeders *Pterostichus nigrita*, *P. augustatus*, *P. oblongopunctatus*, *P. cupreus*, *P. coerulescens* (Thiele, 1966, 1971; Krehan, 1970) and the staphylinids *Tachyporus* spp., *Tachinus* spp. and *Philonthus fuscipennis* (Lipkow, 1966; Eghtedar, 1970) required long days following short days for the full development of the ovaries. In *P. nigrita* maturation followed a clear two-step process. Female beetles emerged in late summer and entered a long-day induced ovarian diapause. The short days of autumn then

brought about the first step (previtellogenesis) characterised by an increase in the activity of the corpora allata and early stages of oocyte development. The second step (vitellogenesis) was associated with the long days of spring and was characterised by a high level of corpus allatum activity and the deposition of yolk in the oocytes (Hoffmann, 1970). Ferenz (1977) showed that the critical day length for step 1 (previtellogenesis) was about LD 15:9, whereas step 2 (vitellogenesis) needed a photoperiod greater than LD 13:11. These insects normally required a sequence from short to long days before vitellogenesis was complete, but with stationary photoperiods, the range from LD 13:11 to LD 16:8 allowed egg maturation without a sequential change (Fig. 10.6). *Pterostichus oblongopunctatus* also needed a sequence from short to long days, but the short-day-induced previtellogenesis (step 1) only occurred between 10 and 15°C (Thiele, 1975; Thiele and Konen, 1975). The autumn breeders *Patrobis atrorufus* and *Nebria brevicollis* (Thiele, 1969, 1971), on the other hand, had an adult reproductive aestivation and required a period of long days and then short days for full maturation.

Tauber and Tauber (1970) provided another example of a response to changes in photoperiod. In the lacewing *Chrysopa carnea* stationary long days (LD 16:8) promoted continuous development and reproduction, whereas short days (LD 12:12) produced a relatively short (about 34 days) imaginal diapause. The critical day length for stationary photoperiods was about LD 13:11. A considerably more intense and durable diapause (about 95 days), however, was brought about by a transfer from LD 16:8 to LD 12:12 during development. More importantly, a transfer from LD 16:8 to LD 14:10 (both above the critical day length) induced a proportion of diapause (29 per cent) among the resulting adults, and a change from LD 8:16 to LD 12:12 (both below the critical) gave 0 per cent diapause. Diapause termination was also effected by such a mechanism: transfer of diapausing adults from LD 8:16 to LD 12:12 caused oviposition to commence in about 16 days, although in stationary photoperiods of 8 and 12 hours the pre-oviposition periods were about 75 and 34 days respectively. These results show that insects can respond to changes in day length even when such changes do not cross the critical value as defined by stationary or 'absolute' photoperiods. In the conifer-inhabiting species *C. downesi*, a series of short days followed by long days was also required for avoiding or terminating reproductive diapause (Tauber and Tauber, 1976).

Several investigators have examined the effects of photoperiods increasing or decreasing at a natural rate. In this type of experiment, care has to be taken to ensure that the changes occur either entirely above, or entirely below, the established critical point, otherwise interpretation becomes difficult. Corbet (1955, 1956) found that the nymphs of the dragonfly *Anax imperator* developed without interruption if they entered their last instar during the spring, but remained in diapause for about 8 months if the last moult occurred after the summer solstice. He pointed out that the same absolute day lengths occurred at both seasons, and attributed the induction of diapause later in the year to decreasing day length. Delayed development in another long-lived insect, *Anthrenus verbasci*, has also been ascribed to decreasing as opposed to stationary photoperiods (Blake, 1960, 1963) (see Chapter 15). In the knot grass moth, *Acronycta rumicis*, shortening day length above the critical photoperiod did not induce diapause, but lengthening the day below the critical appeared to reduce its incidence (Danilevskii, 1965). Other responses to changing day lengths have been demonstrated, or claimed, for *Gerris odontogaster* (Vepsäläinen, 1971), *Crambus tutillus* (Kamm, 1972a), and for the stone-fly *Capnia bifrons* (Khoo, 1968a). Tauber and Tauber (1973b) have shown that diapausing females of *Chrysopa carnea* remain responsive to natural illumination throughout diapause. As the autumn days became shorter (between October 25th and the winter solstice) the rate of diapause development slowed and therefore diapause duration increased. After the winter solstice the time to reactivation got steadily shorter as the season progressed. These

results hardly suggest a simple reaction to absolute stationary day lengths, but rather a continuous sensitivity to photoperiodic changes.

4. Thermoperiod and photoperiod-thermoperiod interactions

Effects of temperature on the photoperiodic response are addressed later in this chapter (see section D1). Here we will consider the diapause-inducing effects of daily temperature cycles, or *thermoperiods*, which may be compared with the other major *Zeitgeber*, or photoperiod. In some species a daily thermoperiod may regulate diapause induction in darkness (or in rare cases, under continuous illumination); in others thermoperiod may have diapause-inducing or averting effects in combination with photoperiod. The latter will be examined first.

Goryshin (1964) showed that the proportion of *Acronycta rumicis*, *Leucoma salicis* and *Pieris brassicae* entering diapause was a function of 'night' rather than 'day' temperature. Working with *A. rumicis* under short-day cycles (LD 12:12 to LD 16:8), he showed that diapause incidence in regimes with days at 30°C and nights at 17°C was almost as high as in the same photoperiods at a constant temperature of 17°C. If the days were at 17°C and the nights at 30°C, however, the incidence of diapause was very much lower, although still above that for 30°C throughout. Lees (1953b) also demonstrated a reduction in the proportion of winter females if the mite *Panonychus ulmi* was maintained in regimes with a warm night. Beck (1962a) showed, for the corn borer *Ostrinia nubilalis* under a light cycle of LD 15:9, that a day temperature of 31°C and a night temperature of 21°C resulted in as much diapause (96 per cent) as a constant temperature of 21°C. On the other hand, a day at 21°C and a night at 31°C gave merely 15 per cent diapause, the same as at 31°C throughout the 24 hours. In the examples given above it is clear that night temperature is more important than day temperature. This is perhaps hardly surprising – at a superficial level – because insects have evolved in an environment in which nights are colder than the days. It may also demonstrate, however, that night-length measurement is 'more important' than day length measurement (see Chapter 11D).

Using the pink boll worm moth *Pectinophora gossypiella*, Pittendrigh and Minis (1971) examined the effects of a light-cycle (LD 8:16) and a concurrent sinusoidal temperature cycle (20° to 29°C daily) in which the phase angle between the two cycles was varied systematically. They too found 'long-day' effects when the low point of the temperature cycle fell during the day and 'short-day' effects when it fell at night. The experiments were designed as a specific test of the 'external coincidence' model and will be discussed more fully in Chapter 13.

A daily thermoperiod is also known to have diapause-inducing properties in the absence of a light cycle. Beck (1962b), for example, maintained larvae of the European corn borer, *O. nubilalis* in continuous darkness but in a temperature cycle consisting of 11 hours at 31°C, 11 hours at 10°C, and the remaining 2 hours in the warming and cooling phases. Such a regime induced nearly all of the larvae to enter diapause, whereas constant temperatures of 31°, 26° and 21°C in darkness caused very few larvae to become dormant. Beck (1968) also quoted unpublished work by D. G. R. McLeod in which 'long-day' thermoperiods (warm phase lasting for 16 hours per day) were found to be "not as effective as long-day photoperiods in the avoidance of diapause". Menaker and Gross (1965) raised larvae of the pink boll worm moth *Pectinophora gossypiella* in continuous darkness and fluctuating temperatures. They showed that a thermoperiod of 12 hours at 31°C and 12 hours at 21°C induced a higher proportion of diapause larvae than a regime consisting of DD and a constant temperature of 26°C (the mean of 31 and 21°C). Using *Acronycta rumicis*, *Pieris brassicae* and *Spilosoma menthastri*,

Goryshin and Kozlova (1967) demonstrated that 'short-day' temperature cycles (12 hours at 26°, to 32°C per day) produced a somewhat higher proportion of diapause than 'long-day' temperature cycles (18 hours at 25° to 29°C per day).

A clear-cut dependence of diapause induction on thermoperiod in darkness was demonstrated for the parasitic wasp *Nasonia vitripennis* (Saunders, 1973a). Wasps were raised from the egg stage in the complete absence of light and then subjected, as adults, to a variety of 'square-wave' temperature cycles obtained by transferring the insects from one incubator (at 23°C) to another (at 13°C). The type of progeny produced (diapause or nondiapause larvae) was then examined during a 2-day 'test period' between the 15th and 17th days of adult life. In short-day thermoperiods (between 6 and 10 hours at 23° per day) almost all of the wasps produced diapausing larvae. On the other hand, diapause was completely absent when the females experienced the warmer conditions for 14 or more hours per day (Fig.10.7). The proportions of females producing diapausing progenies at constant temperatures of 13°C and 18°C (the arithmetic mean of 12 hours at 13°C, and 12 hours at 23°C) were about 44 and 2 per cent, respectively. The 'critical thermoperiod' was observed to be about 13 hours at 23°C per day. This was shorter than the critical photoperiod observed earlier for this species (15½ hours light per 24) (Saunders, 1966a), but the difference was assumed to reflect the slow rate of cooling (and perhaps heating) consequent upon the manner of transfer from one incubator to the other. More abrupt temperature transitions might have produced a critical thermoperiod closer to that for light. In the cabbage butterfly *Pieris brassicae*, Dumortier and Brunnarius (1977a) obtained a well-marked critical thermoperiod in DD; in this case it was close to that for photoperiod. In the south-western corn borer, *Diatraea grandiosella*, on the other hand, the threshold between diapause-inducing short thermoperiods and diapause-averting long thermoperiods was less sharp than that for light and, like *N. vitripennis*, at a different value (Chippendale et al., 1976). Nevertheless, these observations demonstrate that a daily temperature cycle may simulate the diapause-inducing or averting effects of photoperiod in the complete absence of light, although the temperature cycle may be a 'weaker' *Zeitgeber* than photoperiod. Since these important observations have relevance when considering the nature of the clock mechanism, thermoperiodic effects on diapause induction will be considered again in later sections (Chapters 11 and 13).

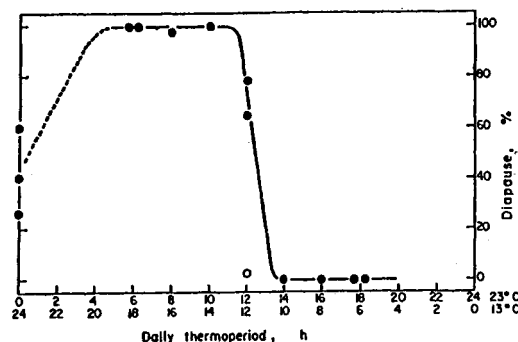


Fig. 10.7. The effects of a daily temperature cycle (thermoperiod; 23°/13°) on the induction of larval diapause by the parasitic wasp, *Nasonia vitripennis* kept throughout in constant darkness, showing a sharp discontinuity between short and long thermoperiods. The ordinate plots the proportion of female wasps producing diapausing broods of larvae during a test period (days 15 to 17 of adult life). The open circle shows the proportion producing diapausing larvae in the dark at 18°C. (From Saunders, 1973a). (Copyright 1973 by the American Association for the Advancement of Science).

Thermoperiodic induction of larval diapause in the European corn borer, *O. nubilalis*, was examined in detail by S.D. Beck in a series of important studies during the 1980s. In 1982 and 1984 he showed that diapause incidence under square-wave temperature cycles and continuous darkness was dependent upon the duration of the cold period of the cycle, or *cryophase*. The critical cryophase was about 9.5 hours, but the temperature during this part of the cycle had to be below about 17.5°C but above 10°C, the threshold for larval development. In later papers (Beck, 1985a, 1987) he described strong interactions between the daily temperature cycle and photoperiod. The temperature and duration of the cool dark period (the 'cryoscotophase') was found to be more important than that of the 'thermophotophase'. Furthermore, a temperature below 10°C during the scotophase caused an increase in the critical night length, and an even lower temperature (0°C) effectively prevented any photoperiodic induction, presumably by 'stopping' the clock. A similar thermoperiodic effect on diapause induction was described for the predacious mite, *Amblyseius potentillae* (Van Houten et al., 1988). In this species, diapause in DD and a constant temperature of 15 to 27°C was either very low or absent, but the addition of a thermoperiod (12 hours at 15° and 12 hours at 27°C) induced a high incidence of diapause. The photoperiodic and thermoperiodic response curves were rather similar in this species, with critical values close to 14 hours/24.

Masaki and Kikukawa (1981) showed that diapause induction in the Indian meal moth, *Plodia interpunctella*, could also be regulated by a daily thermoperiod under *continuous illumination* (LL) (Fig. 10.8). This observation has important consequences for the nature of the clock mechanism regulating diapause in this species, and will be considered further in Chapter 13. Finally, in some species such as the parasitic wasp *Cothonaspis boulandi* (Claret and Carton, 1980), the tufted apple budmoth *Platynota idaeusalis* (Rock, 1983) and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1984) there was no compelling evidence for the thermoperiodic induction of diapause. Larvae of the flesh fly raised in darkness under 'square-wave' thermoperiods (cryophases at 15° and thermophases at 25°) between 4:20 and 20:4 entered diapause at a rate equivalent to that in DD and a constant temperature equal to the arithmetic mean of the cycle.

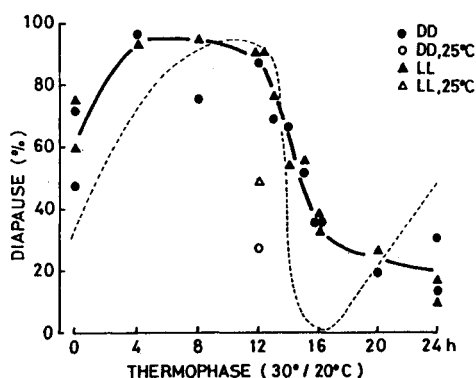


Fig. 10.8. Regulation of larval diapause in *Plodia interpunctella* by thermoperiod (30°/20°) under continuous light (LL) and continuous darkness (DD). The broken line shows the photoperiodic response at 25°C. (From Masaki and Kikukawa, 1981).

B. THE SENSITIVE AND RESPONSIVE STAGES

The period of the life cycle sensitive to photoperiod never extends to the whole of development. In some species the *sensitive period* occurs in the same instar as the resulting diapause, in many others it precedes it. Very frequently, young larvae are photoperiodically sensitive and diapause occurs in the last larval instar. This is observed, for example, in the Oriental fruit moth *Grapholitha molesta* (Dickson, 1949) and the European corn borer *Ostrinia nubilalis* (Beck and Hanec, 1960). Often the larvae are sensitive and diapause supervenes in the pupa; this is seen in the oak silk moth *Antheraea pernyi* (Tanaka, 1950) and in the noctuid moth *Diataraxia oleracea* (Way and Hopkins, 1950). These 'delayed photoperiodic responses' led de Wilde (1962) to distinguish (a) photoperiodic induction, a reversible, partly photodynamic process from (b) photoperiodic determination, the induced state of the overall controlling centre of growth and reproduction, and (c) the photoperiodic response, or the reaction of the effector system.

In some species the sensitive period extends to cover the diapause stage, so that diapause termination, as well as induction, may be governed by photoperiod. These species - for example, *A. pernyi* (Williams and Adkisson, 1964), *O. nubilalis* (McLeod and Beck, 1963), *P. gossypiella* (Bell and Adkisson, 1964), the pitcher plant midge *Metriocnemus knabi* (Paris and Jenner, 1959), the midge *Chironomus tentans* (Engelmann and Shappirio, 1965) and the phantom midge *Chaoborus americanus* (Bradshaw, 1969a) - enter diapause in the autumn when days shorten below the critical value and may resume development in the spring when the critical daylength is exceeded, provided that the temperature is above the threshold for morphogenesis. In *Antheraea pernyi* the critical day lengths for induction and termination are 'mirror images' of each other, suggesting that the same clock process is involved in both (Fig. 10.9). Müller (1970) regards this type of control as photoperiodically induced quiescence, or 'oligopause'.

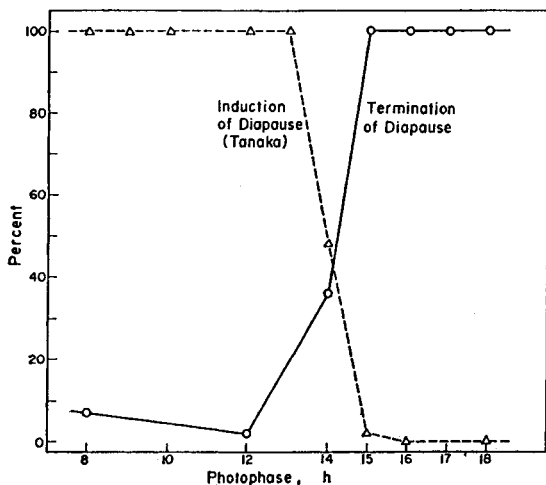


Fig. 10.9. Photoperiodic induction and termination of pupal diapause in *Antheraea pernyi*. Note that the critical day length for termination is a 'mirror image' of that for induction. (From Williams and Adkisson, 1964.)

As indicated above, the sensitive period frequently comes to an end before the diapausing instar. In the flesh flies (Sarcophagidae), for example, larvae are particularly sensitive as embryos within the maternal uterus (Denlinger, 1972; Vinogradova, 1976; Roberts and Warren, 1975; Saunders, 1980b). The larvae may then remain sensitive throughout their development (Saunders, 1971) but become insensitive at the time of puparium formation. The diapause stage is the pupa. In the butterfly *Araschnia levana* the second to fourth larval instars are the most sensitive, but the pupa becomes dormant (Müller, 1955). A more extreme example is provided by the vine-leaf roller, *Polychrosis botrana* (Komorova, 1949). In this species pupal diapause is induced by photoperiod experienced by the eggs and early larval instars. Since the sensitive period comes to an end before the diapause stage, reactivation of diapause is controlled by non-photoperiodic mechanisms, most usually a prolonged period of exposure to low temperature. In Müller's (1970) terminology this constitutes 'true' diapause or 'eudiapause'. However, although diapause cannot be reversed in these species by photoperiodic treatments during the dormant stage, it can be reversed by long days applied during the sensitive period.

C. MATERNAL INDUCTION OF DIAPAUSE AND SEASONAL FORMS

The most extreme examples of a 'delayed' photoperiodic response are to be seen when the sensitive stage occurs in one generation and the arrest of development in the next. Such maternal influences are particularly interesting because they raise a number of important questions about the nature of photoperiodic determination (Chapters 12 and 13).

The classical example of a maternal influence on diapause induction is the commercial silk moth *Bombyx mori*, in which photoperiodic signals experienced by the eggs and young larvae of one generation determine the diapause or nondiapause status of the eggs in the next (Kogure, 1933). It is known that this mechanism involves the production of a diapause hormone by the sub-oesophageal ganglion of the adult female that enters the ovarian egg (see Chapter 9). Embryonic diapause is also maternally induced in the Psocoptera (Glinyanaya, 1975; Eertmoed, 1978).

In a growing number of species, however, photoperiods experienced maternally are known to induce diapause in the larvae of the next generation. Since this form of diapause is most probably of the normal larval-pupal type involving a cessation of activity in the brain neurosecretory cells, this kind of control differs from that in *B. mori*. A few examples of insects with a maternally operating photoperiod will be described here.

Females of the parasitic wasp *Nasonia vitripennis* deposit their eggs within the puparia of 'higher' flies. Under conditions of long days ($>15\frac{1}{4}$ hours per 24) these eggs hatch to give rise to nondiapause larvae which pupate and produce the next generation of adults without interruption. In short-day conditions ($<15\frac{1}{4}$ hours per 24), however, females 'switch-over' from the production of nondiapausing progeny to those which enter a larval (or pre-pupal) diapause late in the fourth instar, just before defaecation and the pupal ecdysis (Saunders, 1965b, 1966a). The type of progeny produced may be reversed during the maternal sensitive period by an appropriate manipulation of the light cycle (Fig. 10.10). Once the eggs have been deposited within the host puparium, however, the type of development (i.e. diapause or non-diapause) is fully determined: it cannot be reversed by exposing the immature instars to different day lengths, or to temperature changes within the ecological range (Saunders, 1966a). The diapausing larvae are also insensitive to photoperiod and require a prolonged period of cold for diapause development and reactivation (Schneiderman and Horwitz, 1958). Topical application of ecdysone will also promote pupation (De Loof et al., 1979). A similar type of

control has been described for the braconid *Coeloides brunneri* (Ryan, 1965) and for the blow flies *Lucilia caesar* and *L. sericata* (Ring, 1967). Pupal diapause in the horn fly, *Lyperosia irritans*, is also dependent on a maternally acting photoperiod (Depner, 1962), as is the egg diapause in a variety of aëdine mosquitoes (Anderson, 1968; McHaffey and Harwood, 1970).

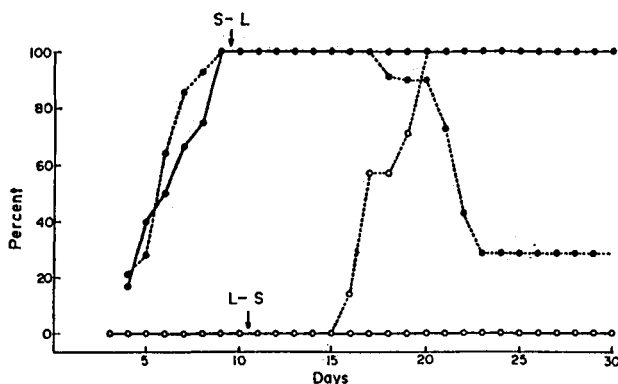


Fig. 10.10. The effect of transferring females of *Nasonia vitripennis* from short day length to long day length (closed circles) or from long day length to short day length (open circles) at the arrows, on the production of diapausing offspring. Ordinate shows the proportion of females producing diapause larvae. This demonstrates the reversibility of the inductive process within the sensitive period. Closed circles, solid line - short days throughout; Open circles, solid line - long days throughout. (From Saunders, 1965b.)

Larval diapause in the blow fly *Calliphora vicina* is also controlled by the maternal light regime (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a). Flies exposed to long days lay eggs giving rise to offspring with no developmental arrest, whereas those maintained under short days (less than 14½ hours per day), at a temperature below about 15°C, produce most of their offspring as diapausing larvae (Saunders, 1987a; Vaz Nunes and Saunders, 1989). As with *N. vitripennis*, an inversion of the photoperiod during the maternal sensitive period resulted in a reversal of the response. It is probable that this type of control is much more widespread than previously realised. For example, the onset of pupal diapause in the cabbage-root maggot, *Erioischia brassicae*, which was formerly attributed to indirect photoperiodic influences transmitted through the tissues of the host plant (Hughes, 1960), is now known to depend, at least in part, on the photoperiods 'seen' by the female progenitor (Read, 1969).

The most complex cases of maternal control occur in aphids. Their extremely rapid reproduction during the summer months is a consequence of their viviparous, parthenogenic habit and the 'telescoping' of successive generations. A reproducing virginopara of *Megoura viciae*, for example, may contain the early embryonic stages of her grandchildren. In this species the type of progeny produced (virginoparae or oviparae) is determined by the photoperiod acting on the parent virginopara (Lees, 1959). The photoperiodic centre in the brain (Chapter 14) begins to function about 2 to 3 days before the birth of the parent but the first embryos do not become responsive until the parent has developed to the second instar. These embryos are only sensitive when they are in the second and third positions in the embryonic chain; before this they cannot respond, and after this they are fully determined as either virginoparae or oviparae. During the summer months when days are longer than about 14½ hours all the progeny are produced as a further generation of virginoparae. When the

photoperiod falls below the critical point, however, oviparae (and males) are born, the former proceeding to deposit diapausing eggs. Close to the critical day length parent aphids switch from the production of daughter virginoparae to oviparae and back again, in a 'flip-flop' fashion, indicating an unstable equilibrium in the maternal controlling centre. Because of the 'telescoped' development the interval between the sensitive period and the resulting egg diapause can be regarded as spanning three generations.

Since diapause-inducing short days occur twice per year, in spring as well as autumn, long-day species could theoretically enter a maladaptive diapause in the spring if no alternative or preventative mechanism were to operate. Such mechanisms might include determination of the *direction* of photoperiodic change (see section A3) or the absence of the sensitive stages in the spring. Another has been described for the flesh fly *Sarcophaga bullata* (Henrich and Denlinger, 1982). They showed that flies emerging from diapausing pupae (i.e. the first spring generation) were incapable of producing diapausing progeny themselves, even under strong short day conditions. This block to diapause was a result of short-day exposure of the mothers whilst they were intra-uterine embryos, and normal responses to short days only reappeared after a further generation under long days. Subsequent investigation (Rockey et al., 1989) suggested that a maternal 'message' was passed from the brain to the ovaries sometime between the end of larval life and the third day of adult life, and that haemolymph and CNS factors were probably involved. Two-dimensional gel electrophoresis of the ovaries of flies (*S. crassipalpis*) raised under long or short days (Denlinger et al., 1995) revealed a unique mRNA expressed only in the ovaries of females with a short day history, i.e. those which produce progeny that will *not* enter diapause. Expression of this maternal effect in other flesh flies is variable. In *S. crassipalpis*, it is present but less pronounced than in *S. bullata* (Henrich and Denlinger, 1982). In *S. argyrostoma*, it appears to be absent, at least in strains from northern Europe (Kenny et al., 1992). It may be that more northerly flesh flies have a longer pupal diapause so that appearance of the first post-diapause generation of flies is delayed until photoperiods exceed the critical value.

D. FACTORS WHICH MODIFY THE PHOTOPERIODIC RESPONSE

1. Temperature

Temperature may affect the expression of the photoperiodic clock and the induction of diapause in a number of ways. (1) *Constant* temperatures frequently modify the degree of the response, and may alter the position of the critical day length. (2) Low- or high-temperature *pulses* may completely reverse the photoperiodic response, from short to long or long to short, depending on the time in the light/dark cycle at which the pulse is applied. (3) Single temperature *steps* (up or down) may apparently alter the 'stored photoperiodic information' at the end of the sensitive period (Gibbs, 1975). Finally, (4) a daily temperature *cycle*, or thermoperiod, may simulate most or all of the effects of a light-cycle, by acting as a *Zeitgeber* in its own right. The diapause inducing effects of thermoperiod have been discussed above (section A4); here we will consider the other effects of temperature on the photoperiodic response.

The results of a large number of investigations with long-day species have shown that a high constant temperature and long day length act together to avert diapause, whereas low temperature and short day length act together to induce it. Similarly, high temperatures may facilitate diapause termination in those species reactivated by long day length. In short-day species, however, an opposite effect has been noted: in the commercial silk moth, *Bombyx*

mori, for example, low temperatures reduced the diapause-inducing effect of long days, whereas high temperatures enhanced it (Kogure, 1933). High temperatures also promoted summer diapause in the geometrid *Abraxas miranda* (Masaki, 1958).

Figure 10.11 shows the modifying effects of temperature on the photoperiodic responses of a number of insect species. In the cabbage white butterfly *Pieris brassicae* a short day (LD 12:12) was fully inductive (100 per cent diapause) up to about 25°C. At higher temperatures the proportion of pupae entering diapause dropped sharply so that at about 30°C all of the insects developed without arrest (Danilevskii, 1965). Pupal diapause in the tomato moth *Diataraxia oleracea* was fully expressed in short days at temperatures below 30°C; above this the incidence of diapause dropped (Way and Hopkins, 1950). Similarly in the flesh fly *Sarcophaga argyrostoma* under LD 10:14, practically all of the larvae became diapausing pupae at 15° to 18° but there was a drop at 20°, and at 25°C and above the short-day response was eliminated (Fig. 10.12) (Saunders, 1971). In the silk moth *Antheraea pernyi* the photoperiodic response was at one time thought to be virtually independent of temperature (Tanaka, 1944). A more recent investigation of this species (Mansingh and Smallman, 1971), however, showed that the same 'rule' applies. At temperatures of 24° to 26°C short days induced about 100 per cent of pupal diapause, at 28° to 30°C it was 94 per cent, but at 32° the proportion of dormant pupae dropped to 32 per cent. In some long-day species, normally diapause-averting (long) photoperiods induce diapause if the temperature is sufficiently low. A model to account for these temperature effects is developed in Chapter 12.

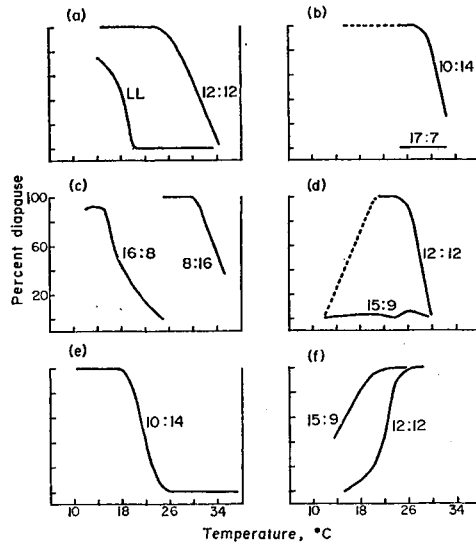


Fig. 10.11. The effects of temperature on the incidence of diapause at short and long day length. A - *Acronycta rumicis* (after Danilevskii, 1965); B - *Antheraea pernyi* (after Mansingh and Smallman, 1971); C - *Diataraxia oleracea* (after Way and Hopkins, 1950); D - *Grapholitha molesta* (after Dickson, 1949); E - *Sarcophaga argyrostoma* (after Saunders, 1971); F - *Bombyx mori* (after Kogure, 1933).

The oriental fruit moth *Grapholitha molesta* shows a more complex relationship between temperature and diapause. In this species short-day induction only occurred within the range 20° to 27°C; diapause was avoided at both higher (30°C) and at lower (12°C) temperature (Dickson, 1949).

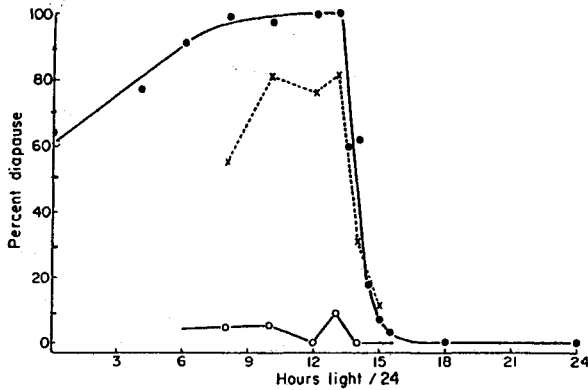


Fig. 10.12. The effect of temperature on the photoperiodic response in *Sarcophaga argyrostoma*. Closed circles - 15 °C; crosses - 18 °C; open circles - 25 °C. (From Saunders, 1971.)

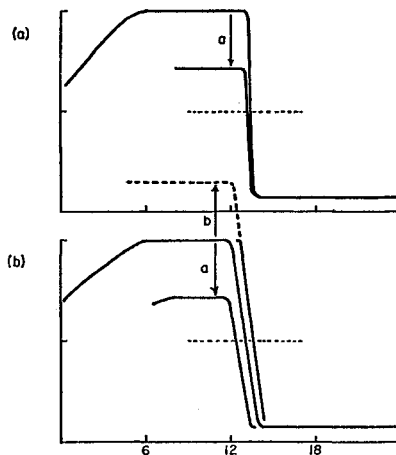


FIG. 10.13. The effect of temperature on the photoperiodic response: theoretical considerations. (a) - raising temperature; (b) - lowering temperature. In (a), a species with a steep critical day length, raising the temperature may reduce the amount of diapause at short day length, but has little effect on the position of the critical photoperiod. In (b) a species with a less steep critical day length, altering the temperature may have a more pronounced effect on the position of the critical day length. These effects may be confused with those shown in Fig. 10.1, A and C.

Constant temperatures are also known to affect the 'position' of the critical day length (see Fig. 10.1). In some insects, such as *Pieris brassicae*, temperatures up to about 26°C had little effect on the critical photoperiod (Danilevskii, 1965). This species, therefore, was regarded as having a temperature-compensated response over practically the entire range of ecologically important temperatures. The critical day length in the aphid *Megoura viciae* shifted to shorter values by about 15 minutes for every 5°C rise in temperature, but at 23°C and over, short-day induction of oviparae failed completely (Lees, 1963). The European corn borer, *Ostrinia nubilalis* (Beck and Hanec, 1960), similarly showed a rather slight effect of temperature on the critical point: at 19°C it was only about 30 minutes longer than at 29°C.

In other species the critical photoperiod decreased steadily as the temperature increased. In *Acronycta rumicis*, for example, it moved to shorter values by about 1½ hours with every 5°C increment (Goryshin, 1955). In the spinach-leaf miner *Pegomya hyoscyami* and the cabbage-root maggot *Erioischia brassicae* the critical day length shortened by 3 and 4½ hours, respectively, with a 7°C rise in temperature (Zabirov, 1961).

The question of temperature-compensation of the critical day length is one of importance since it may affect the date at which natural populations of insects begin to enter diapause in the autumn. At a physiological level, however, the manner in which temperature affects the response is far from clear. It could, for example, affect the 'clock' mechanism itself, or it could modify the response at a more superficial level. Figure 10.13 demonstrates that an overall reduction of the diapause response caused by an elevated temperature could result in a shortened critical value without necessarily affecting the mechanism of time measurement. Conversely a lowered temperature might cause an increase in the critical day-length. Figure 10.13 also shows that the degree of the change might be a function of slope of the curve at the critical point. Such effects have not been demonstrated with certainty, but data on the critical day length in *Pectinophora gossypiella* after selection for 'early' and 'late' eclosion strains (Chapter 13) indicate its possibility (Pittendrigh and Minis, 1971).

Short 'pulses' of high or low temperature may have spectacular effects on the diapause response; in some insects complete reversals have been reported. For example, working with the larvae of *Acronycta rumicis*, Danilevskii (1965) showed that chilling at 5°C for 3 hours daily at the beginning, or at the end, of the light period in LD 17:7 converted the response from a long to a short day. However, chilling the middle of a 17-hour light period, or similar periods of chilling in the dark component of the cycle, had no such reversing effects. Danilevskii concluded that low temperature was equivalent to darkness: insects were unable to 'see' the light at 5°C. In plants, reversals of the photoperiodic control of flowering in the short-day plant *Xanthium pensylvanicum* by chilling were reported by de Zeeuw (1957) and by Nitsch and Went (1959). Schwemmler (1960) also reported reversals by chilling in *Hyoscyamus niger* and *Perilla ocymoides*. These authors, like Danilevskii, considered that chilling in the light had a similar effect to that of darkness. Danilevskii (1965) also showed that brief periods of heating applied at the end of the night in an LD 13:11 cycle produced a drop in the diapause response provided that the pulse was above about 38°C.

In the parasitic wasp *Nasonia vitripennis* photoperiodic reversals were achieved by chilling and by heating at different phases of the light/dark cycle. In a cycle of LD 14:10, which is just short of the critical daylength (15¼ hours per 24), a daily period of 4 hours at 2°C applied at the beginning or in the middle of the long night converted the response from diapause-inducing to diapause-averting (Saunders, 1967, 1968, 1969). Chilling during the light component of this cycle merely had a 'strengthening' effect on the short-day response. In the converse experiment at LD 16:8, chilling in the dark had no effect, but chilling in the light reversed the response from a long-day (diapause-averting) to that of a short-day (diapause-

inducing). In a very short-day cycle of LD 8:16 - which is well short of the critical day length - no such reversals were observed. These results differed from those obtained by Danilevskii (1965) for *A. rumicis* in that both 'night' and 'day' phases were sensitive to a period at low temperature, so that, following his argument, the period at low temperature could be 'seen' either as dark or light depending on the phase-point at which it is experienced. An alternative explanation may be that the clock mechanism is stopped or phase shifted by the low-temperature pulse so that long days and long nights were effectively shortened. Subsequent experiments with 3-hour daily periods of chilling (at 2°C) or heating (at 35°C) at all points of the 'clock' showed that both forms of treatment had a similar effect on the response of *N. vitripennis* in LD 14:10 (Saunders, unpublished) (Fig. 10.14). In particular it was observed that maximum photoperiodic reversal occurred when the low- or high-temperature pulse was placed at the beginning or at the end of the 10-hour 'night'; chilling or heating in the middle of the night gave a smaller response.

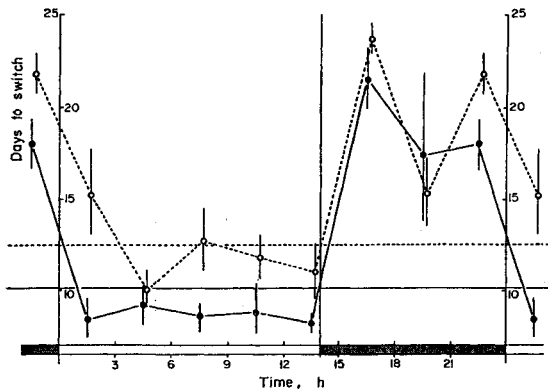


Fig. 10.14. The effects of daily periods of chilling (3 hours at 2 °C, solid circles) or heating (3 hours at 35 °C, open circles) on the photoperiodic response of adult females of *Nasonia vitripennis*, otherwise kept at 18°C and short daylength (LD 14:10). The plotted points show the mean number of days to the 'switch' to diapause larva production; the vertical lines show 2 x SE of the mean. The horizontal dotted line shows the unheated control, the solid line the unchilled control. Note that both chilling and heating have maximum diapause-averting (long-day) effects at the beginning and end of the night.

Beck (1985a) showed that night length measurement in the corn borer *Ostrinia nubilalis*, was strongly influenced by a low temperature pulse (0°C) placed early in the dark phase where it effectively stopped the clock. Working with the green vetch aphid, *Megoura viciae*, Lees (1986) subjected the insects to a 4 hour low temperature pulse at the beginning of the night, before returning them to 15°C to assess their measurement of night length. He found that night length measurement was almost perfectly temperature compensated between 6° and 20°C, but temperatures lower than 6°C caused the clock to run more slowly. It was still running at -3°C, but at only a quarter of its rate. Chilling (down to -3°C) during the light component of the cycle apparently interfered with photoreception, the chilled portion of the photophase being interpreted as darkness.

Single step-wise transfers of insects from a higher to a lower temperature, or vice versa, may also have an effect on the photoperiodic reaction. Working with *Sarcophaga argyrostoma*

raised in diapause-inducing short days (LD 10:14), Gibbs (1975) collected the 'diapause-committed' pupae daily and subjected them to a step-up to a higher constant temperature, or to a step-down to a lower temperature, all within a few hours of puparium formation. Steps up caused a decrease in the incidence of pupal diapause, and steps down an increase, the degree of the change being a function of the size of the step and the time after puparium formation. Since puparium formation in this species is regarded as the end of the 'sensitive period' (Saunders, 1971) and the experimental treatment involved no photoperiodic changes, the temperature steps were considered to affect the accumulated or 'stored' photoperiodic 'information'. These experiments will be considered again later with reference to the photoperiodic 'counter' mechanism (Chapter 12).

2. Diet

Insect diets rarely remain the same throughout the year; they frequently change with the season, both in quality and quantity. Plant-eating insects such as lepidopteran larvae or aphids, for example, may have access to young green leaves in the spring and early summer, but only to yellowing foliage in the autumn. Those feeding on fruits and seeds are subjected to chemical changes associated with maturation and ripening. Predacious insects are subjected to seasonal changes in both the number and type of prey available. In a number of insects these qualitative and quantitative changes in nutrition are known to affect diapause induction and the expression of the photoperiodic mechanism. As with temperature, diet usually modifies the degree of the photoperiodic response or occasionally the position of the critical day length. In some insects, however, diet may provide a major influence in the seasonal cycle of activity.

One of the best-known examples of nutritional quality affecting the photoperiodic response is afforded by the pink boll worm moth, *Pectinophora gossypiella* (Adkisson, 1961; Bull and Adkisson, 1960, 1962; Adkisson et al., 1963). Larvae of this species were raised on artificial diets containing different quantities of fats and oils. Those raised at 27°C and short day length (LD 10:14 to LD 12:12) produced only about 15 per cent diapause on diets containing 0.25 per cent of wheat-germ oil, but almost 80 per cent diapause on diets containing 5 per cent of cotton-seed oil. This difference was thought to reflect natural increases in oil contents as the bolls ripened. In this example the degree of the response to short day length was clearly modified by diet. In another cotton pest, *Chloridea obsoleta*, however, a diet of cotton leaves - as opposed to cotton bolls - caused a shift in the critical day length from about 13½ to about 14½ hours (Danilevskii, 1965). As with the rather similar effects of temperature, modifications of the degree of the response and the photoperiodic threshold may be aspects of the same phenomenon.

Lees (1953a) showed that females of the red spider mite *Panonychus ulmi* laid mainly nondiapause eggs when reared on young apple foliage under long days. Those transferred to yellowing leaves, however, produced about 68 per cent of winter eggs. The same effect was observed when the mites were supplied with foliage which had been 'bronzed' by previous and heavy infestations of *P. ulmi*. The white-fly *Aleurochiton complanatus* also showed a tendency to produce an increased proportion of diapause stages (winter 'puparia') when fed on yellowing foliage (Müller, 1962b). In a few instances the species of plant is known to have an effect: Danilevskii (1965), for example, found that the larvae of the beet web worm *Loxostege sticticalis* developed without arrest on pigweed (*Chenopodium album*), but showed a high incidence of diapause on wormwood (*Artemisia inana*).

Several publications have demonstrated the importance of the presence of food, or the quantity of food. Termination of larval diapause in the phantom midge *Chaoborus americanus*,

for example, required the action of long days *and* the presence of a source of food such as mosquito larvae (Bradshaw, 1969a, 1970). When brought in from the field, diapausing larvae of this species never developed to the pupal stage if starved and kept under short day length, although short-day insects supplied with prey showed a variable degree (2 to 49 per cent) of reactivation. Under conditions of long day length, however, 4 to 50 per cent of the larvae pupated if starved, but nearly all of them (92 to 98 per cent) terminated diapause if provided with an abundance of prey. A synergistic action of long days and food was apparent when both components were present. Clay and Venard (1972) found that the incidence of larval diapause in *Aedes triseriatus* increased when short-day larvae were provided with an inadequate diet (less than an optimum quantity of pulverised dog food) or were kept at low temperature. In this case both inadequate food and low temperature exerted their effect by slowing larval development (see Chapter 12).

Tauber and Tauber (1973a) described an effect of diet on the incidence of diapause in the lacewing *Chrysopa mohave*, which feeds on insects such as aphids during its larval and adult instars. Under short-day conditions (<14 hours light per 24) the adults were found to enter a reproductive diapause that was terminated by exposure to photoperiods in excess of 16 hours. However, withholding prey also induced the diapause syndrome - even in adults maintained in long days - and supplying prey terminated it. Natural populations of this insect in California were found to reproduce during April, May and June when the days were long and prey abundant, but some insects were found to enter a 'food-mediated' summer diapause when food was in short supply. In October, November and December the whole population entered a short-day-induced reproductive diapause that enabled them to overwinter.

In the parasitic wasp *Nasonia vitripennis* withholding host puparia - on which the adult wasps feed as well as deposit their eggs - is known to modify the response to short day length (Saunders, 1966b). Female wasps maintained in LD 12:12 at 18°C and supplied each day with two host puparia (*Sarcophaga argyrostoma*), produced about 73 per cent of their progeny as diapausing larvae. Those deprived of hosts for the first 3, 5 and 7 days of adult life, however, produced about 86, 91 and 99 per cent diapausing progeny, respectively.

In a number of early studies it was suggested that the seasonal cycles of phytophagous insects were influenced, or even controlled, by chemical changes associated with the photoperiodic responses of the plant host. This view was particularly prevalent with root-feeding insects that were not exposed directly to daily cycles of illumination. In his work with the strawberry root aphid, for example, Marcovitch (1924) was inclined to believe that the observed photoperiodic response was mediated through the tissue of the plant on which the insects were feeding. In more recent years this has been suggested for the cabbage-root maggot *Erioischia brassicae* (Hughes, 1960), and even for the aphid *Megoura viciae* (von Dehn, 1967) which feeds above ground. Lees (1968), however, pointed out that the strawberry-root aphid tends to feed at or near ground level where access to light might occur. He countered von Dehn's suggestion with the observation that a prenatal sensitivity to photoperiod occurs in *M. viciae* (Lees, 1967b), and that differences in the manner in which the parents of the experimental aphids were kept could therefore account for von Dehn's results. In the case of *E. brassicae*, subsequent investigations by Read (1969) also demonstrated a maternal sensitivity to day length. Furthermore, several authors, including Way and Hopkins (1950) with *Diataraxia oleracea* and Lees (1953a) with *Panonychus ulmi*, made unequivocal demonstrations that arthropod photoperiodic reactions are independent from those of their host plants.

Interactions between the seasonal cycles of insect parasites and that of their hosts do seem to occur, however. An extensive literature has built up on this subject mainly concerned

with the problem of whether diapause is independently induced by environmental factors, or whether it is more or less influenced by the diapause state, or other physiological characteristics of the host species. Maslennikova (1968) has demonstrated that examples of both may occur.

Rearing the braconids *Apanteles glomeratus* and *A. spuria* in a variety of Lepidopterous hosts, Geispitz and Kyao (1953) showed that photoperiod acted directly and independently upon the parasites. Under short day length the parasites entered diapause within the body of the host whereas under long day length they emerged and pupated within their cocoons. Maslennikova (1958) confirmed this independent sensitivity to day length in *A. glomeratus*. Nevertheless she was able to demonstrate a modifying influence of the host species. When this braconid parasitises larvae of the univoltine black-veined white butterfly *Aporia crataegi* it hibernated within the body of its host as a first instar larva and adopted the host's univoltine life-cycle. In the multivoltine species *Pieris brassicae*, however, the parasite was also multivoltine, the correspondence in the number of generations being due to a similarity between the independent photoperiodic reactions of the two species.

Vinogradova and Zinovjeva (1972a) found similar effects on the braconids *Aphaereta minuta* and *Alysia manducator*. These species attack the larvae of various blow flies and flesh flies but complete their development within the flies' puparia. Larvae of the flesh fly *Parasarcophaga similis* were raised in long-day conditions, parasitised by *A. manducator*, and then exposed to either long-day (LD 20:4) or short-day (LD 12:12) photoperiods. In the long-day group about 15 per cent entered diapause, whereas in the short-day group about 82 per cent entered diapause; this demonstrated that the induction of diapause in the parasite was to a large extent independent of the host. On the other hand, when the experiment was repeated with host larvae raised in short-days (i.e. with 'diapause-committed' hosts), 43 per cent of the parasites entered diapause after subsequent long-day treatment, whereas nearly 80 per cent did so after short days. This experiment demonstrated an influence of the host's diapause condition upon that of the parasite. With the gregarious parasitoid *Aphaereta minuta*, Vinogradova and Zinovjeva (1972a) showed that host species modified the parasites' reaction to photoperiod. This braconid was raised in a variety of blow fly and flesh fly larvae subjected to different day lengths. Under long days (LD 20:4) and 18°C almost all of the parasites emerged from their hosts by the 60th day, regardless of the host species involved. But under a short-day regime (LD 12:12) the wasps emerged quickly from *Parasarcophaga similis*, less quickly from *P. (= Sarcophaga) argyrostoma* and *Calliphora vicina*, but very slowly from *Bellieria melanura*.

In the parasitic wasp *Nasonia vitripennis* larval diapause was controlled by the photoperiod experienced maternally (Saunders, 1966a). Female wasps maintained at short day length (<15¼ hours light per 24) switched to the production of diapausing larvae after a few short day cycles, but those exposed to long day length produced nearly all their progeny as nondiapausing broods. Furthermore, once the eggs were deposited within the puparia of the host (Cyclorrhaphous flies) subsequent development could not be modified by exposure to an opposing photoperiod, or to changes in temperature. The species of host used, however, did modify the response to short days (Saunders et al., 1970). With *Sarcophaga argyrostoma* as host, the life-span of the adult wasps was relatively short (21 to 27 days), but the switch to the production of diapausing progeny occurred fairly early in adult life (8 to 11 days) so that the proportion of diapause larvae produced was high (65 to 75 per cent). With puparia of *Calliphora erythrocephala* (= *vicina*) or *Phormia terraenovae*, however, the wasps had a longer life (34 to 58 days), but the switch was delayed so that the final proportion of diapause larvae was lower (30 to 48 per cent). This difference was attributed, in part, to qualitative differences associated with the haemolymph on which the adult wasps had fed. For example,

wasps supplied with different host species on alternate days showed an intermediate rate of switching to the production of diapause larvae as if the production of diapause progeny was being modified by a mixed diet consisting of the haemolymph of the two species. If the nutritional differences were affecting the larvae directly, a different incidence of diapause would have been found on alternate days.

2. Density effects

Crowding of insects on their food may also influence diapause incidence, although it is not always possible to distinguish density-dependent effects from those of nutritional quality. Sometimes crowding appears to act directly as a factor for diapause induction; in other cases it modifies responses to photoperiod and temperature. A few examples will be mentioned here. In the sycamore aphid *Drepanosiphum platanoides*, short days led to the production of autumnal sexual forms that laid diapausing eggs. However, a second post-hibernal generation also entered diapause during the summer months in response to poor quality sycamore leaves and crowding during nymphal development (Dixon, 1975). Among stored product insects such as *Ephestia cautella*, larval overcrowding induced diapause apparently because dense cultures become tainted, perhaps with a pheromone (Hagstrum and Silhacek, 1980). Likewise, in the codling moth, *Laspeyresia pomonella*, larval density strongly influenced diapause incidence even during the long days of summer (Brown et al., 1979), and prolonged crowding induced larval diapause in the drosophilid *Chymomyza costata* (Botella and Mensua, 1987). Working with a Japanese strain of the migratory locust, *Locusta migratoria*, Tanaka et al. (1993) showed that crowding of the hoppers, together with long days, led to a delayed sexual maturation of the ensuing adults.

The blow fly *Calliphora vicina*, on the other hand, showed a situation in which larval overcrowding *reduced* the incidence of diapause (Saunders, 1997). When larvae produced by short-day exposed adults, and therefore directed along the diapause pathway (Saunders, 1987a), were severely over-crowded within a limited amount of food, the smallest larvae 'side-stepped' the diapause 'programme' to become miniature (nondiapausing) puparia and then adults, despite the continuing short days. It was postulated that avoidance of diapause allowed under-sized individuals an opportunity to reproduce before the winter set in. Saunders et al. (1999) showed that very small larvae were much less cold tolerant than their full-sized siblings, perhaps lending credence to this 'bet-hedging' hypothesis.

E. GEOGRAPHICAL CLINES IN PHOTOPERIODIC TRAITS

The length of the day and its rate of change are functions of latitude. After the spring equinox, days are longer at higher latitudes and day length increases more rapidly than in areas further south. With an increase in latitude, however, the climate is usually colder, thus restricting the length of the breeding season: at higher latitudes the short summer comes to an end earlier and whilst the days are still long. Even at the same latitude the climate is generally harsher at an increased altitude, or in areas well removed from the influence of an ocean. Insects have responded to these geographical variations in climate by evolving appropriate modifications to their photoperiodic responses.

Initially, Russian entomologists dominated the study of geographical variation, probably because their national boundaries encompassed such a vast area and such extremes of climate. Danilevskii (1965) and his associates, for example, investigated the photoperiodic

responses of a number of species, particularly Lepidoptera, from the south (40°N) to the north (60°N), and from the warm climate of the Black Sea to Siberia.

An increase in *latitude* was shown to be associated with an increase in the critical day length (Fig. 10.16). In the knot grass moth *Acronycta rumicis*, a population from the Black Sea coast (Sukhumi, 43°N) showed a critical value of about 14½ hours per 24, whereas populations from Belgorod (50°N), Vitebsk (55°N) and Leningrad (St Petersburg) (60°N) showed critical photoperiods of 16½, 18 and 19½ hours, respectively. These data showed that the critical day length changed by about 1½ hours with every 5° of latitude. Associated with this change in the critical day length was also a change in the number of generations per year (voltinism). Southern populations were usually bivoltine or multivoltine with a facultative winter diapause. The St Petersburg population, on the other hand, was univoltine and the photoperiodic response curve showed a distinct tendency towards an obligate form of diapause since a proportion of the pupae entered diapause even at day lengths in excess of 20 hours. The selective advantage of this was clear: insects at higher latitude compensated for the longer summer day length and the earlier onset of winter with a higher diapause threshold. Southerly populations, on the other hand, were able to exploit the longer growing period by delaying the

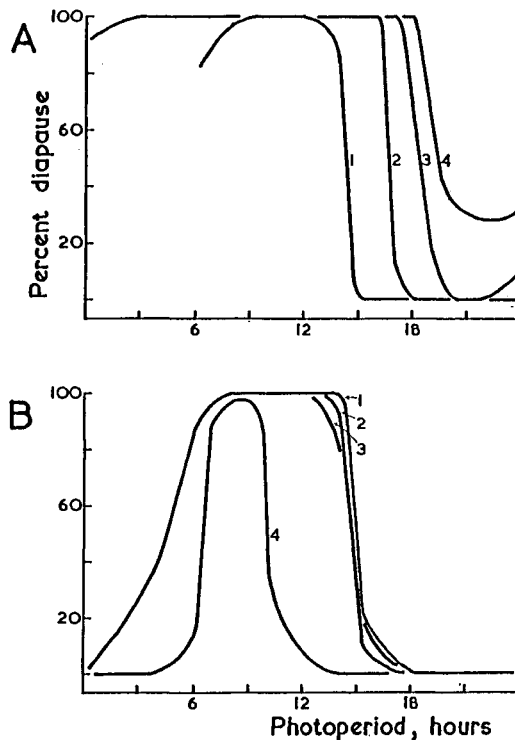


Fig. 10.15. The effects of latitude on the photoperiodic response, for two Russian species of insects. (a) *Acronycta rumicis* at (1) Sukhumi 43°N, (2) Belgorod 50°N, (3) Vitebsk 55°N, (4) St Petersburg 60°N. (b) *Pieris brassicae* at (1) St Petersburg 60°N, (2) Brest-Litovsk 52°N, (3) Belgorod 50°N (4) Sukhumi 43°N. (From Danilevskii, 1965.)

onset of diapause with a shorter critical photoperiod. The ecological realities of these differences were demonstrated by transferring populations from one latitude to another. Southern races transferred to St Petersburg, for example, failed to diapause and died with the first frosts. Northern races transferred to the Black Sea, on the other hand, entered diapause after only one generation, even though the environment would have supported a second.

Similar data were described for a number of other species. The cabbage moth *Mamestra brassicae*, showed a critical day length of about 14½ hours on the Black Sea coast, but about 18½ hours near St Petersburg (Danilevskii, 1965). In Japan, this species was bivoltine throughout its distribution, but its phenology was complicated by the appearance of a summer diapause in southern latitudes (Masaki, 1956, 1968). Thus, in Hokkaido (41° to 45°N) the moth completed two generations during the relatively short breeding season and overwintered in a pupal diapause. In northern Honshu (40°N) some pupae had a brief period of summer aestivation. This summer diapause increased in intensity and duration in more southerly latitudes until in the Ryukyu islands (28°N) the insect, although still bivoltine, was active only in the spring and the autumn, and spent the winter (November to March) and the summer (May to September) in diapause.

In Eastern Europe both *A. rumicis* and *M. brassicae* form continuous series, or clines, from south to north with no clearly defined geographical races. In the cabbage butterfly, *Pieris brassicae*, on the other hand, the critical day length was the same (about 15 hours per 24) in St Petersburg (60°N), Brest-Litovsk (52°N) and Belgorod (50°N). On the Black Sea coast (43°N), however, a distinct geographical race occurred with a critical photoperiod of about 10 hours per 24 (Danilevskii, 1965). These races appeared to be genetically distinct.

Examples of geographical races or clines with respect to the photoperiodic reaction are now known in a large number of insect species (Beck, 1968; Danilevskii et al., 1970; Tauber, Tauber and Masaki, 1986; Table 10.1). Important examples include the following. The lacewing, *Chrysopa carnea*, showed a critical photoperiod of 13.5 to 14 at Ithaca, New York (42°27'N), but 12.5 to 13 in Arizona (33°19'N) (Tauber and Tauber, 1972b). Working in North America with the pitcher-plant mosquito, *Wyeomyia smithii*, Bradshaw (1976) measured critical day lengths for twenty-two populations collected over 19° of latitude, 20° of longitude, and 1200 m of altitude. Critical day lengths were closely correlated with latitude and altitude, but not with longitude. They were also correlated with the length of the 'growing season' (mean number of freeze-free days). Bradshaw calculated that latitude accounted for 80.5 per cent of the variation in critical photoperiod, and altitude for 15.5 per cent. Jordan and Bradshaw (1978) studied populations of *Aedes sierrensis* from a number of localities between southern California (33°N) and Oregon (44°N). The critical day length was found to increase by 1 hour for every 4.8° of latitude, and at 16.5°C, short days induced 100 per cent larval diapause in Oregon and northern California but only 35 per cent in the south. Similar data have been found for the Psocopteran *Peripsocus quadrifasciatus* and the codling moth *Laspeyresia pomonella*, again in North America. In *P. quadrifasciatus*, which shows a maternally induced embryonic diapause, fifteen populations were studied from Quebec (48°40'N) to Alabama (31°10'N) (Eertmoed, 1978). The critical day length in the most northerly population was about 14½ hours/24 and that for the most southerly population about 10 hours/24. There was a linear dependency of critical photoperiod on latitude with an increase of about 15 minutes for every degree of latitude. Latitude accounted for about 86 per cent of the variation and altitude for about 4 per cent. In *L. pomonella* there was a similar relationship between latitude and critical day length among sixteen populations collected from Michigan (43°50'N) to New Mexico (33°25'N) (Riedl and Croft, 1978). In the most extensive study, Lankinen (1986a) studied the

photoperiodic responses of *Drosophila littoralis* collected from 57 localities ranging from the Black Sea coast (41°N) to northern Finland (69°N). Critical day lengths were shown to range from about 12 hours in the south to over 19 hours in the north with a high degree of correlation ($r = 0.943$) (Fig.10.16).

TABLE 10.1. Representative examples of latitudinal (south to north) clines in photoperiodic/diapause traits.

Species	Trait	No. strains	Latitudinal range (S→N)	Clinal variation (S→N)	Reference
<i>Acronycta rumicis</i>	CDL	4	43 → 60°N	14.4→ 19.5 h/24	Danilevskii (1965)
<i>Mamestra brassicae</i>	CDL	4	ditto	14.5→ 18.5 h/24	ditto
<i>Pieris brassicae</i>	CDL		Ditto	2 distinct races	ditto
	voltinism				Masaki (1968)
<i>Chrysopa carnea</i>	CDL	2	33 → 42°N	12¾→13¾ h/24	Tauber & Tauber (1972b) Nechols et al. (1987)
	diap. int.			deeper in south	
<i>C. oculata</i>	CDL	10	25 → 50°N	11½ → 14 h/24	
	diap. int.			deeper in north	
<i>Ostrinia nubilalis</i>	CDL	3	32 → 45°N	14 → 16h/24	Takeda & Skopik (1985)
	voltinism	7	37 → 45°C	bi- → univoltine	Beck & Apple (1961)
<i>Nasonia vitripennis</i>	CDL	2	42 → 52 °N	13¼→15¼ h/24	Saunders (1966a)
<i>Pectinophora gossypiella</i>	CDL		27°S→32°N	none	Ankersmit & Adkisson (1967)
<i>Wyeomyia smithii</i>	CDL	22	30 → 49°N	increases to N	Bradshaw (1976)
<i>Aedes sierrensis</i>	CDL		33 → 44°N	increases to N	Jordan & Bradshaw (1978)
<i>Peripsocus quadrifasciatus</i>	CDL	15	31 → 49°N	10 → 14½ h/24	Eertmoed (1978)
<i>Laspeyresia pomonella</i>	CDL	16	33 → 43°N	14¼→15½ h/24	Riedl & Croft (1978)
<i>Diatraea grandiosella</i>	CDL	2	19 → 37°N	13 → 15 h/24	Kikukawa & Chipendale(1983)
<i>Drosophila littoralis</i>	diap. int.				
<i>D. auraria</i>	CDL	57	41 → 69°N	12 → 19.1 h/24	Lankinen (1986)
	CDL	3	34 → 43°N	12 → 14h/24	Pittendrigh & Takamura (1987)
<i>Tetranychus urticae</i>	CDL	10	40.5 → 60°N	10¾→ 16¼ h/24	Vaz Nunes et al. (1990)
	diap. int.	8	ditto	deeper to north	Koveos et al. (1993)
<i>Dianemobius spp</i>	various	several	28 → 44°N	various	
<i>Teleogryllus spp</i>					
<i>Calliphora vicina</i>	CDL	5	36 → 65°N	12½ → 15 h/24	McWatters & Saunders (1996)
	diap. int.	3	44 → 65°N	deeper to north	Saunders (2000)
	cold tol.	3	44 → 65°N	greater to north	Saunders (2000)
					Saunders & Hayward (1998)

South-north clines in diapause intensity (see Chapter 9) were described for the southwestern corn borer, *Diatraea grandiosella* (Kikukawa and Chippendale, 1983), the spider mite, *Tetranychus urticae* (Koveos et al., 1993) and the blow fly *Calliphora vicina* (Saunders, 2000b) in which a longer lasting, deeper diapause occurred in more northerly populations. On the other hand, in the lace-wing *Chrysopa carnea*, diapause intensity was greater in a more southerly population (Tauber and Tauber, 1972b).

Table 10.1 lists representative examples of latitudinal clines. In most cases, more northerly populations show a longer critical day length, fewer generations per year, a more intense diapause and an increased tolerance of the cold. In the far south of a species' range there may be a tendency towards continuous or homodynamic growth, whereas to the extreme north there may be an opposite tendency towards a univoltine life cycle with an 'obligate' diapause.

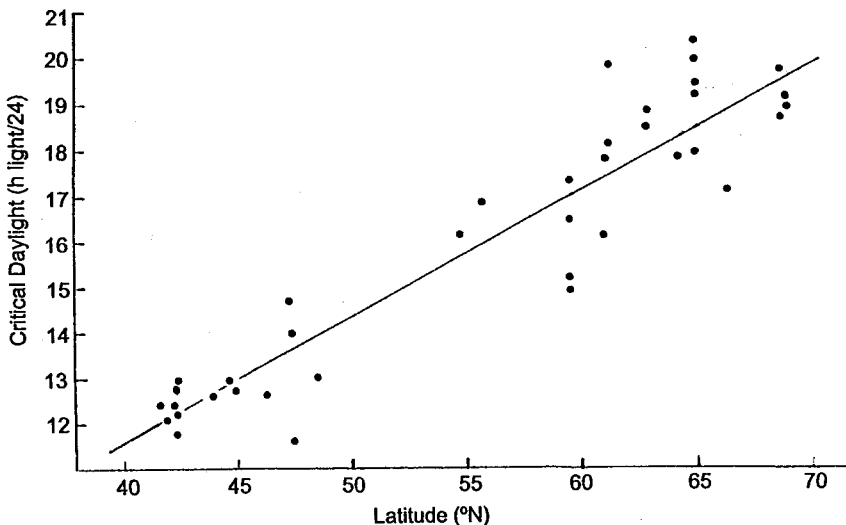


Fig. 10.16. *Drosophila littoralis*. Critical day lengths (CDLs) for the induction of ovarian diapause in 38 populations from a north-south cline, from Kilpsijärvi, Finland (69°N) to Batumi, Georgia (41.6°N). CDLs varied from over 20 hours in the north to about 12 hours in the south. Data from Lankinen (1986a).

Of particular interest is the phenological adaptation of recently introduced species to local conditions. The European corn borer, *Ostrinia nubilalis*, for example, was introduced into North America and first recorded in New Hampshire in 1921. Beck and Apple (1961) showed that populations collected from different areas now show different critical photoperiods, different numbers of generations per year, and different rates of larval growth. More recently, Takeda and Skopik (1985) studied strains of *O. nubilalis* from three localities within the United States: a northern population from Minnesota (MN), a coastal population from Delaware (DE) and a southern population from Georgia (GA). The induction of larval diapause was then investigated a range of photoperiods (LD 10:14 to LD 16:8) at two constant temperatures (20° and 30°C). The critical day length (CDL) was found to be both strain and temperature dependent. In all three strains the CDL was longer at the lower temperature, and the northernmost strain (MN) showed a greater tendency to enter diapause at both temperatures. In a later paper, Skopik and Takeda (1987) showed that thermoperiodic

induction (see section A4) and photoperiodic termination of diapause were both strain dependent in *O. nubilalis*. Thus, dormant larvae of the northern population (MN) responded more slowly to diapause terminating photoperiod (LD 16:8) than did GA, and only MN responded positively to the diapause inducing effects of a thermoperiod (12 hours at 25° and 12 hours at 4°C) under constant light.

Unlike the corn borer, the pink boll worm moth *Pectinophora gossypiella*, has retained remarkably constant photoperiodic traits since its introduction into the Americas early in the present century. Ankersmit and Adkisson (1967) studied populations of this insect from Texas (32° and 28°N), the Virgin Islands (18°N), Venezuela (10°N), Colombia (3°N) and Argentina (27°S). In all of these localities the critical day length was about the same (12 to 13 hours/24), although the intensity of the response was more pronounced in the most northerly and most southerly populations. In the tropical populations (Venezuela and Colombia) the photoperiodic response was weak and almost eliminated at high temperature. The Colombian strain produced a high incidence of diapause at LD 10:14 but this photoperiod is never encountered at that latitude. The photoperiodic responses of the tropical populations, therefore, were rarely operating, and the insect was showing a strong tendency towards a homodynamic type of development.

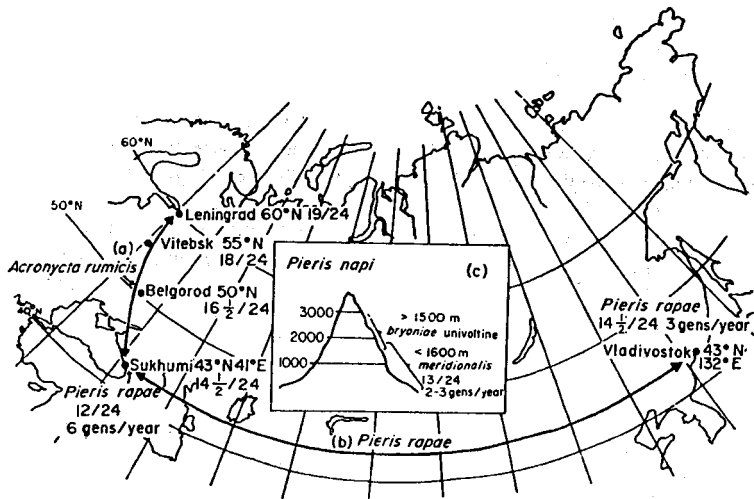


Fig. 10.17. The effects of latitude (a), longitude (b), and altitude (c) on the diapause response (critical day length and number of generations per year) in *Acronycta rumicis*, *Pieris rapae* and *P. napi* in Russia. (Data from Danilevskii, 1965.)

Geographical differences in the photoperiodic response at different localities *at the same latitude* have been investigated in a number of species (Fig. 10.17). The small cabbage white butterfly *Pieris rapae*, for example, accomplished six generations a year on the Black Sea coast and showed a critical day length of less than 12 hours/24. However, at Vladivostok - which is almost at the same latitude but is subjected to a much longer and more severe winter - the same species only managed three generations a year and entered diapause at a critical day length of 14½ hours/24 (Danilevskii, 1965). *Altitude* puts similar constraints on the number of

generations achieved because of the retarded development at lower temperature. The white satin moth *Leucoma salicis* achieved one generation per year above 2000 m but two generations on the plains. In the Caucasus, the green-veined white butterfly, *Pieris napi*, occurred in two distinct forms associated with altitude. Up to about 1600 m there is a genetically uniform race (ssp. *meridionalis*) with a 13-hour critical photoperiod and several generations a year. Above about 1500 m, however, there is a univoltine and phenologically separate subspecies (*bryoniae*) which has a very rudimentary photoperiod response only observed at high temperature.

Most of the photoperiodic responses reviewed above are for insects inhabiting higher latitudes where season, particularly an adverse winter, is well marked, and changes in day length relatively large. In sub-tropical and tropical areas with annual dry seasons, however, diapause and other dormancies are still common, even in areas close to the equator where changes in day length are absent or only slight (see Chapter 9, section 2).

The grasshopper *Oedipoda miniata* has an aestival diapause in the Israeli dry season (Orshan and Pener, 1979). The females enter a reproductive diapause and the males show little mating behaviour when photoperiods are relatively long and temperature high, but diapause is broken in the autumn. In North Africa (Libya and Morocco), another area characterised by long, dry summers, many beetles enter an aestival dormancy but breed in the cooler and wetter winter when the photoperiod falls below about 10 hours per day (Paarmann, 1977). In these areas annual changes in day length are still about 3 to 4 hours, and clearly sufficient to act as a seasonal synchroniser.

Further south and within the tropics themselves, changes in day length become progressively smaller and the role of photoperiod in controlling seasonal patterns less clear. Some long-lived insects, such as the locust *Nomadacris septemfasciata*, are able to sense the sequential change of day length from short to long, or from long to short, even when living within 10° of the equator (Norris, 1959, 1962) (Chapter 9, A.2). Other species appear to rely on environmental cues other than photoperiod. Paarmann (1977), for example, examined the seasonal breeding patterns of several beetles in an area of Zaire where annual photoperiodic changes spanned a mere 16 minutes; these species showed a gonadal dormancy during the short dry season (June to August) apparently in response to the slight changes in temperature.

F. THE GENETICS OF THE PHOTOPERIODIC RESPONSE

The foregoing sections clearly demonstrate that the photoperiodic response and the diapause-associated traits that it controls are genetically 'programmed' in the development of the organism. Thus, in a particular species, diapause occurs at a specific stage in the life cycle. Photoperiodic responses - particularly the length of the critical photoperiod, the proportion of the population entering diapause and the intensity of diapause - are also evolved characters matching the local conditions of photoperiod and temperature in the area from which the population was drawn.

Genetical and evolutionary aspects of photoperiodism, diapause and associated traits have received extensive treatment in a number of books and reviews (see, for example, Tauber, Tauber and Masaki, 1986; Taylor and Karban, 1986; Brown and Hodek, 1983; Dingle, 1984). Such studies on photoperiodism and diapause fall into two main categories. The first depends on the intrinsic genetic variability of natural or laboratory populations and involves the selection for either diapause or diapause-free lines. The second approach involves the cross mating of individuals from different geographical populations with different diapause and photoperiodic characteristics. Examples of each will be given here.

1. Selection for low and high diapause

The spruce budworm *Choristoneura fumiferana* is a univoltine species that enters diapause as a second-instar larva within a silken hibernaculum. It is typical of all insects, however, in that its genome contains a considerable 'store' of genetic variability. Thus Harvey (1957) was able to select an almost diapause-free line in only six generations by breeding from the few insects which failed to diapause, and thereby to isolate a strain with a facultative rather than an obligate form of arrest. Similarly, Barry and Adkisson (1966) maintained the pink boll worm *Pectinophora gossypiella* at short day length (LD 12:12) and 28°C, and bred from the few non-diapausing insects; after twenty-three generations an almost diapause-free strain was obtained. Tanaka (1951), on the other hand, bred only from the diapausing individuals of *Antheraea pernyi* and developed a strain showing univoltine characters. In some cases selection has taken place inadvertently: House (1967), for example, recorded a gradual loss of diapause through successive laboratory-reared generations of the parasitoid *Pseudosarcophaga affinis*. Of particular interest is the selection of a short-day strain of *A. pernyi* from an originally long-day population by breeding from individuals which completed only one generation a year (Chetverikov, quoted in Danilevskii, 1965). In this case a complete inversion in the photoperiodic response was achieved which shows that the long- and short-day responses probably only differ in the 'clock-controlled' processes linking time measurement with the endocrine control mechanisms.

Tauber, Tauber and Masaki (1986) list over 40 cases of artificial selection of the diapause trait; only a few of these will be considered here. Notable examples of divergent selection for and against diapause include those for the moth *Pionea forficaris* (King, 1974), the melon beetle *Atrachya menetriesi* (Ando, 1978), the flesh fly *Sarcophaga bullata* (Henrich and Denlinger, 1983) and the pitcher plant mosquito *Wyeomyia smithii* (Istock et al., 1976). Selection for low diapause, sometimes inadvertent, was achieved for the pentatomid *Aelia acuminata* (Honek, 1972), the gypsy moth *Lymantria dispar* (Hoy, 1977) and the fruit fly *Drosophila littoralis* (Oikinaren and Lumme, 1979). Selection for high diapause was also achieved for the cricket *Pteronemobius fascipes* (Masaki, 1978), the blow fly *Calliphora vicina* (Vinogradova, 1975b; Saunders, 2001) and the fly *Delia antiqua* (Robinson et al., 1980). These studies indicate that natural populations of insects contain a large amount of variability in diapause-associated traits, and that both a low or a high incidence of diapause could be achieved after rather few generations of selection.

Figure 10.18 (top) shows the selection of high and low diapause strains from a laboratory population of the blow fly *C. vicina* in which the incidence of larval diapause had been considerably weakened over years of culture under LL at 25°C. From this 'parental' stock, which showed only about 20 per cent diapause under short days (LD 12:12 at 16°C), a low diapause line was selected by breeding from the first larvae to pupariate in each generation, and a high diapause line by breeding from those with a delayed pupariation. After only two generations, larval diapause was eliminated from the former, and after five generations diapause was restored in the latter to 90 per cent. Photoperiodic response curves (PPRCs) for the stock, low and high lines are shown in Fig. 10.18 (bottom).

2. Crossing strains with different photoperiodic and diapause characteristics

Cross mating strains from different geographical areas was performed by Danilevskii (1965) for *Acronycta rumicis*, using races isolated in Leningrad (St Petersburg) (60°N) and in

Sukhumi (43°N). At 23°C the St Petersburg strain showed a critical day length (CDL) of about 19 hours/24 and the Sukhumi strain a critical day length of about 15 hours/24. First-generation (F_1) crosses between these two races gave rise to larvae showing an intermediate CDL of about 17 hours/24. The inbred progeny of these individuals (the F_2) also showed a 17-hour critical photoperiod. Danilevskii pointed out that this threshold was equivalent to that for a population of *A. rumicis* drawn from a latitude of about 50°N, and considered that these results indicated a continuous gradient of gene frequencies from south to north with continuous hybridisation.

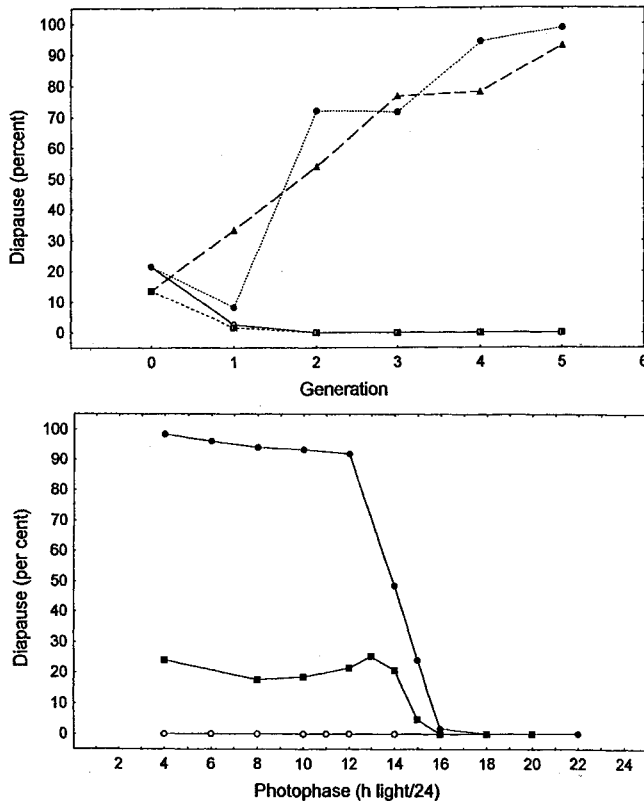


Fig. 10.18. Top – artificial selection for high incidence of larval diapause (closed points) and low incidence of diapause (open points) (two parallel cultures of each) from a diapause depleted stock of *Calliphora vicina*. Bottom – photoperiodic response curves (PPRCs) for *C. vicina* from the 5th generation of selection for high (closed circles) and low (open circles) incidence of larval diapause, compared with the PPRC for the 'parental' diapause-depleted stock. All experiments under LD 12:12 at 16°C. (From Saunders, 2001).

When the F_1 progeny were analysed according to sex, it was found that the male offspring of either cross (St Petersburg female x Sukhumi male, or Sukhumi female x St Petersburg male) showed a critical photoperiod closer to that of the female parent (Fig. 10.19). Female offspring, on the other hand, showed critical values strictly intermediate (about 17 hours) between the parental types. The reaction of the male hybrids therefore clearly inclines towards the maternal characteristics.

Although all crosses between these widely separated geographical strains were achieved without difficulty and the progeny developed quite normally, there was clear evidence of 'hybrid vigour', or heterosis. In the parental strains the duration of larval development was about 20 to 21 days in short day length and about 19 days in long. The F_1 hybrids, however, developed more rapidly: in short day length larval development was completed in about 18 days and in long days in about 16 days. Larval weights also showed evidence of heterosis. Sukhumi larvae were usually larger than those from St Petersburg, female larvae of the former weighing about 310 mg as opposed to about 274 mg. Female larvae derived from crossing the two races, however, weighed about 320 mg.

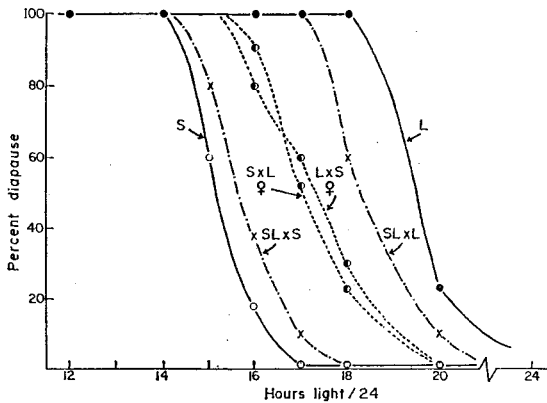


Fig. 10.19. The results of crossing experiments between strains of the moth *Acronycta rumicis* from two different latitudes. The critical day length for the strain from Sukhumi (S) on the Black Sea coast (43°N) was about 15 hours; that for a strain from St Petersburg (L) (60°N) was about 19 hours. The critical day lengths for the females of both F_1 crosses (S x L and L x S) were about 17 hours, and the back-crosses between these hybrids and moths of the original strains (SL x S and SL x L) were similarly intermediate in respect of their critical day lengths. Not all the crosses are shown, and only the females of the F_1 are included. The males resulting from these particular crosses showed a critical day length closer to the maternal type. These results show both the genetic nature of the photoperiodic response and that inheritance is multifactorial. (From Danilevskii, 1965.)

The photoperiodic responses of larvae resulting from back-crosses to the parental strains were also investigated. In this type of cross the response was identical for either sex and showed a critical day length intermediate between the two parents. Thus, a cross between a female of the Sukhumi x St Petersburg strain (with a critical day length of 17 hours) and a male of the St Petersburg strain (about 19 hours) gave progeny with a critical photoperiod close to 18 hours. Similarly, a cross between a Sukhumi x St Petersburg female (17 hours) and a Sukhumi male (15 hours) gave progeny with a 16-hour threshold. The absence of segregation in these back-crosses indicated that the genetic control of the photoperiodic response is polygenic.

Crosses between geographical races have now been performed in a number of other insect species. Russian entomologists, for instance (see Danilevskii, 1965), have demonstrated a similar intermediate photoperiodic response in F_1 hybrids of *Spilosoma menthastris*, *Pieris brassicae* and *Leucoma salicis*. In the parasitic wasp *Nasonia vitripennis* the rate of switching to the production of diapause larvae was investigated in crosses between strains from

Cambridge, England (52°N), and Woods Hole, Massachusetts (42°N)(Saunders, 1965b). In conditions of constant darkness females of the English strain switched to all-diapause broods more rapidly than those from Massachusetts. When females of one strain were mated with males of the other the pattern of diapause production in the larvae so produced was identical to the maternal type, confirming that diapause induction in this species is determined in the adult female. Crosses between these offspring, however, revealed the F₁ type with an intermediate rate of switching to diapause. In this species, therefore, the males only contribute towards the diapause characteristics of their grandchildren and subsequent generations.

Like *N. vitripennis*, larval diapause induction in the blow fly *Calliphora vicina* is maternal in origin (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a). In reciprocal crosses between a southern strain (England, 51°N, with a CDL of 14.5 h/24) and a northern strain (northern Finland, 65°N, with a CDL of 16 h/24), McWatters and Saunders (1996) showed - under an intermediate photoperiod (LD 15.5:8.5) - that the incidence of larval diapause was identical, in each case, to that of the *maternal* population. On the other hand, the *duration* of larval diapause was affected by *both* parents: larvae with northern mothers and southern fathers entered diapause at the same rate as pure-bred northern larvae, but emerged from diapause much sooner. Crosses and back crosses between hybrids of the northern and southern strains later showed that males of *C. vicina* were not only unable to influence diapause incidence among their immediate progeny, but indirect effects down the male line were weaker than those down the female line (McWatters and Saunders, 1997).

G. THE SPECTRAL SENSITIVITY AND THE INTENSITY THRESHOLD OF THE PHOTOPERIODIC RESPONSE

In principle, an experimentally observed action spectrum should correspond directly with the absorption spectrum of the involved pigment molecule. Action spectra were thus used to recognise and to isolate the plant pigment phytochrome (Borthwick et al., 1952). Proper action spectra should also plot the energy (ideally the reciprocal number of quanta in the wavelength interval) required to obtain a particular constant biological response (e.g. 50 per cent diapause) against wavelength. This procedure has been carried out with very few insects, the best-documented example being the aphid *Megoura viciae* (Lees, 1966b, 1971a). Nevertheless, a number of workers have investigated the spectral sensitivity and the light intensity thresholds of the photoperiodic response in insects, at least partially, with a view to the characterisation of the pigment(s) involved. These studies show that most species are optimally sensitive to light in the blue-green region of the spectrum and largely insensitive to red. In some species, however, sensitivity extends into the red end of the spectrum. There is evidently, therefore, a considerable diversity in the pigments involved.

Representative examples of insects that are not red-sensitive are shown in Table 10.2. These species generally show a maximum sensitivity between 400 and 500 nm and a sharp cut-off at longer wavelengths.

In his study of *M. viciae*, Lees (1966b, 1971a) used narrow-band filters (average bandwidth 7 nm at half-peak transmission) in conjunction with a series of neutral density filters. Test virginoparae were exposed to a 1-hour pulse of monochromatic light placed in two positions in the dark phase. The first was 1½ hours after dusk (i.e. LD 13½:1½:1:8), the second a 30-minute pulse 7½ hours after dusk (i.e. LD 13½:7½:½:2½), these two positions corresponding to an 'early' and a 'late' light break (see Chapter 11, C), either of which induced the production of daughter virginoparae. Unbroken nights of 10½ hours (LD 13.5:10.5), or

nights pulsed with an ineffective or sub-threshold stimulus, on the other hand, gave rise to oviparae. The intensity of the light at each wavelength was adjusted in different experiments until a 50 per cent response (50 per cent virginopara producers) was obtained. The results for the early night interruption showed that maximum sensitivity was in the blue (450 to 470 nm) with threshold intensity at that wavelength of about $0.2\mu\text{W cm}^{-2}$ (Fig. 10.20). Fifty times that energy was required to give a 50 per cent response at 365 nm, or in the green at 550 nm; there was no sensitivity in the red. For the late-night interruption, maximum effectiveness was again in the blue, but sensitivity extended some way into the red.

TABLE 10.2. The Spectral Sensitivity of the Photoperiodic Response

Species	Effective wave lengths (nm)	Non-effective wave lengths (nm)	Reference
1. Red-insensitive species			
<i>Bombyx mori</i>	350 – 510	> 600	Kogure (1933)
<i>Grapholitha molesta</i>	430 – 580	< 430; > 600	Dickson (1949)
<i>Panonychus ulmi</i>	365 – 540	> 600	Lees (1953a)
<i>Dendrolimus pini</i>	violet – green	red	Geispitz (1957)
<i>Pieris brassicae</i>	violet – green 400 – 520	red > 580	Geispitz (1957) Claret (1972)
<i>Antheraea pernyi</i>	398 – 508 400 – 500	> 580	Williams et al. (1965) Hayes (1971)
<i>Carpocapsa pomonella</i>	400 – 500	> 600	Norris et al. (1969)
<i>Adoxophyes orana</i>	460 – 480		Berlinger & Ankersmit (1967)
<i>Euscelis plebejus</i>	365 – 550	> 550	Müller (1964)
<i>Anthonomus grandis</i>	blue – orange	red	Harris et al. (1969)
<i>Chaoborus americanus</i>	peak at 550		Bradshaw (1969)
<i>Sarcophaga argyrostoma</i>	peak at 570		Saunders (unpub.)
<i>Megoura viciae</i>	peak at 450 – 470	> 550	Lees (1966, 1971, 1981)
2. Red-sensitive species			
<i>Acronycta rumicis</i>	407 – 655		Geispitz (1957)
<i>Leptinotarsa decemlineata</i>	423 – 674		De Wilde & Bonga (1958)
<i>Pectinophora gossypiella</i>	480 – 680		Pittendrigh et al. (1970)
<i>Nasonia vitripennis</i>	480 – 640	> 650	Saunders (1975a)
<i>Pimpla instigator</i>	386 – 700		Claret (1973)

Williams et al. (1965) studied the termination of pupal diapause in *Antheraea pernyi* by providing 8 hours of white light supplemented with 8 hours of filtered light. Reactivation was found to be rapid with 398, 434 and 508 nm, but longer wavelengths (580 and 650 nm) were inactive. Hayes (1971) similarly showed that this species was optimally sensitive wavelengths between 400 and 500 nm. In *Euscelis plebejus*, Müller (1964) provided filtered light as a short supplementary photoperiod and adjusted the distance from the light source to produce an

irradiance of $160 \mu\text{W cm}^{-2}$, well above the threshold. Maximum sensitivity was between 365 and 550 nm resulting in the production of the long-day or *plebejus* form. Wavelengths above 550 nm were not 'seen' and the *incisus* form was produced.

A pronounced red sensitivity has been found in five insect species (Table 10.2). Larvae of *Acronycta rumicis* were found to be responsive to all three spectral regions (407, 530 and 655 nm) at the energy levels used (Geispitz, 1957). De Wilde and Bonga (1958) exposed the Colorado potato beetle *Leptinotarsa decemlineata* to a constant energy level of $2.0 \mu\text{W cm}^{-2}$ transmitted through narrow-band interference filters. The beetles responded to all wavelengths between 423 and 675 nm, but were unaffected by those greater than 675. Larvae of the pink

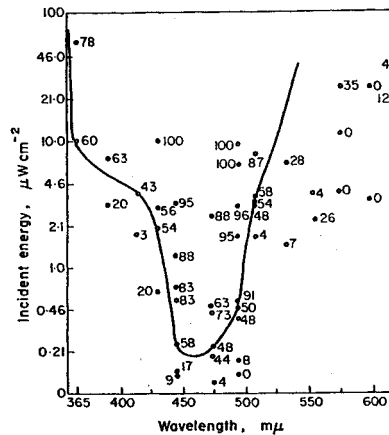


Fig. 10.20. Action spectrum for the photoperiodic control of progeny type in the aphid *Megoura viciae*. Semilogarithmic plot showing the response to 1-hour interruptions of monochromatic light introduced into a 10.5-hour dark period 1.5 hours after its inception. The 'main' photoperiod of 13.5 hours was of white light (100 ft-candles). The intensity of the response is given by the percentage of aphids that produced viviparous progeny. These values are also shown by their respective points and the curve is drawn at approximately the 50 per cent response level. (From Lees, 1966.)

bollworm *Pectinophora gossypiella* could also 'see' red light of low intensity. Pittendrigh et al. (1970) demonstrated that this insect could distinguish (in a photoperiodic sense) between 14 hours of red light per day (640 nm, $0.59 \text{ ergs/cm}^2/\text{sec}$) and 12 hours of red light per day (620 nm, $0.57 \text{ ergs/cm}^2/\text{sec}$). The former was 'read' as a long day and gave rise to only 5 per cent diapause, whereas the latter was 'read' as a short day and gave rise to 98 per cent diapause. Exposure of the larvae to 14 hours of 680 nm light at $0.14 \text{ ergs/cm}^2/\text{sec}$ induced 17 per cent diapause in contrast to the 73 per cent in DD; evidently the spectral sensitivity in *P. gossypiella* extends at least as far as 680 nm. These studies were of particular interest because they suggested that the photoperiodic clock is distinct from that controlling some of the overt behavioural rhythms studied in this species (Chapter 13). The action spectrum for the initiation of the egg-hatch rhythm, for example, showed a maximum effectiveness between 390 and 480 nm and a sharp cut-off above 520 nm (Bruce and Minis, 1969). The ichneumonid *Pimpla instigator* also responded to wavelengths as long as 700 nm (Claret, 1973).

The parasitic wasp *Nasonia vitripennis* distinguished between 12 and 18 hours of red light per day ($>600 \text{ nm}$) and produced 89.3 and 1.8 per cent diapausing progeny, respectively (Saunders, 1973b). Wasps also distinguished between 12 hours of white light per day (100 per

cent diapause) and 12 hours of white light supplemented with 6 hours of red (> 640 nm) (5.7 per cent diapause). Preliminary observations on the action spectrum for this species have been carried out supplying filtered light as a 3-hour supplement following a 13-hour white photoperiod (Saunders, 1975a). A light pulse in this position is regarded as the lights-off signal that entrains and phase-sets the 'dusk' oscillator (Chapter 13). Maximum sensitivity occurred between about 554 and 586 nm with a sharp cut-off above about 650 nm. At 586 nm the wasps responded to less than $1 \mu\text{W cm}^{-2}$.

Although the work of Geispitz (1957) and Claret (1972) indicated that larvae of the cabbage white butterfly *Pieris brassicae* were maximally sensitive to wavelengths between 400 and 520 nm, and insensitive to red (above 580 nm), other observations with the same species produced apparently conflicting results. Bünning and Joerrens (1960) and Bünning (1960), for example, showed that whilst 10 hours of white light per day (LD 10:14), or 2 hours of white supplemented by 8 hours of blue, were diapause inductive, 2 hours of white supplemented by 8 hours of red light were not. Similarly, whilst 16 hours of white light per day (LD 16:8), or 12 hours of white supplemented by 4 hours of blue, eliminated diapause, 12 hours of white supplemented by 4 hours of red light did not. Bünning's (1960) interpretation was that red light was inhibitory (in a diapause-inducing sense) during the first 12 hours of the circadian cycle (i.e. the 'photophil'; see Chapter 11), but promotive during the second half, or 'scotophil'. On the other hand, it might simply be that red light, unlike white or blue, lies outside the absorption maximum and is not 'seen' by the larvae; in this sense, therefore, red light is equivalent to darkness. Work on the same species by Vuillaume et al. (1974), however, conflicted, at least in part, with this interpretation. Thus, although short days (LD 9:15), in which the light portion of the daily cycle was white, U.V., blue (maximum at about 450 nm), green (maximum at about 530 nm), or yellow (maximum at about 590 nm) gave a high incidence of pupal diapause, 9 hours of red light (maximum at 660 nm) gave none. This again suggests that red light is not 'seen'. On the other hand, 9 hours of white light coupled with 15 hours of red (i.e. the 15 hours of darkness in LD 9:15 replaced by red light) reduced diapause incidence from over 90 per cent to about 4 per cent. Clearly in this experiment, red light when given at night was 'seen' and reversed the diapause-inducing effects of the 9 hours of white light. Although there are important differences between the experimental designs of Bünning (1960) and Vuillaume et al. (1974) - principally in that the latter involves red light illuminating the end of the subjective night - the results reviewed above are apparently in conflict and merit careful reinvestigation.

TABLE 10.3. Approximate Intensity Thresholds for the Photoperiodic Reaction

Species	Intensity, in lux	Reference
<i>Bombyx mori</i> , eggs and larvae	0.1 – 0.8	Kogure (1933)
<i>Acronycta rumicis</i> , larvae	5	Danilevskii (1948)
<i>Diataraxia oleracea</i> , larvae	10	Way & Hopkins (1950)
<i>Panonychus ulmi</i>	10 – 20	Lees (1953a)
<i>Leptinotarsa decemlineata</i> , adults	0.1	De Wilde & Bonga (1958)
<i>Metriocnemus knabi</i> , larvae	0.025	Paris & Jenner (1959)
<i>Antheraea pernyi</i> , brain	< 10.8	Williams et al. (1965)
<i>Grapholitha molesta</i> , larvae in young apples	10 – 30	Dickson (1949)

The data obtained from spectral sensitivity studies indicate that the intensity threshold for the photoperiodic response may be quite low. The threshold of *M. viciae* at 450 to 470 nm, for example, is about 0.2 to $0.25 \mu\text{W cm}^{-2}$ (Lees, 1966, 1971, 1981). Other observations, often

with white light, support this. Table 10.3 shows that there is a low threshold value (between 0.025 and 25 lux, depending on the species) above which the receptors appear to be saturated so that they respond in an identical fashion to low light intensities and to full sunlight. In most cases the threshold is above that of the full moon (0.7 lux; $0.65 \mu\text{W cm}^{-2}$) so that moonlight probably does not affect the photoperiodic response. The thresholds, however, are generally below that of the available light at dawn and dusk so that the 'natural photoperiod' is probably closer to the time which includes the two periods of civil twilight (de Wilde, 1962).

The question of intensity thresholds is complicated by the fact that the photoperiodic receptors appear to be in the brain (Chapter 14) so that light reaching the brain has to pass through the cuticle of the head capsule. This must reduce the intensity and probably alter the spectral quality of the light where broad-band filters are used. In addition, many insects live in 'cryptic' situations (e.g. within fruit) which must also modify the quantity and quality of the light eventually reaching the receptors. Larvae of the midge *Metriocnemus knabi*, for instance, respond to light energies down to 0.025 lux but normally live in the water collecting within the pitcher plant *Sarracenia purpurea* (Paris and Jenner, 1959). Conversely, larvae of the oriental fruit moth *Grapholitha molesta* show a threshold of about 10 to 30 lux whilst burrowing in young apples (Dickson, 1949); the light actually reaching the larvae must be much less than this. For example, the threshold for the reactivation of diapausing larvae of the codling moth *Carpocapsa pomonella* - now outside the fruit - was found to be less than $0.1 \mu\text{W cm}^{-2}$ at 450 nm (Hayes, 1971).

Takeda and Masaki (1979) investigated the threshold intensities of light at dawn and dusk in *Hyphantria cunea*. Larvae were exposed to natural daylight of 14 hours 50 minutes (just above the critical photoperiod) in such a way as to include different portions of either the natural dawn or dusk twilights. Threshold intensity levels were then recorded when the 14 hours 50 minutes of light gave a 50 per cent diapause response. A marked asymmetry was observed with the dawn threshold being at about 1 lux and the dusk threshold at about 10 lux, equivalent to a natural photoperiod commencing about 40 minutes before sunrise and ending about 20 minutes after sunset. This asymmetry, which is consistent with observations from action spectra (Lees, 1971a; Saunders, 1975a), is probably a feature of the photoreceptor and its dark adaptation.

Williams et al. (1965) investigated the intensity of light actually reaching the brain of *Antheraea pernyi*. Diapausing pupae of this species are enclosed in an apparently opaque cocoon; the pupal cuticle is also heavily pigmented apart from a small transparent 'window' overlying the brain itself. The transmission of light through the cocoon was measured with a spectrophotometer for a range of wavelengths. At 400 nm it was found to be only 0.000009 per cent of the incident light, and only 0.014 per cent at 700 nm. The transmission of blue light (460 nm) by the transparent facial cuticle was found to be between 2 and 5 times that of the pigmented cuticle elsewhere on the pupa, which in turn was about 5000 times as transparent as the cocoon. A combination of these measurements on the cocoon and the facial cuticle suggested that as little as 0.0000003 per cent of the blue light (460 nm) falling on the outside of the cocoon could reach the brain by simple transmission. The geometry of the cocoon in *A. pernyi*, however, apparently made it an ideal 'light-integrating sphere'. It collects scattered light, especially in the spectral range 440 to 510 nm, within it as a blue haze. In the effective range (440 to 490 nm) more than 0.14 per cent of the light energy reached the brain which was fully saturated by less than 10.8 lux of this blue light.

The transparent facial window referred to earlier, however, may only play a minor role in the transmission of light to the brain. Shakhbazov (1961) reported that photoperiodic responses of *A. pernyi* could be eliminated by coating the window with an opaque material.

Williams (1969a), however, failed to repeat this observation: the photoperiodic responses of pupae with their windows painted black and returned to their almost opaque cocoons were fully preserved. Williams concluded that the transparent facial cuticle was merely a "safety device for cocoons in shady situations".

The pronounced blue-sensitivity of many insect species has suggested that the photoperiodic pigment is either pink (Williams et al., 1965) or orange (Lees, 1966). However, both in *M. viciae* and in *A. pernyi* the brain appears colourless to visual inspection, and this suggests that the pigment is in a very low concentration. These authors, and others, have suggested that the chromophore might contain a carotenoid. Pteridine pigments have also been proposed as a possible candidate (L'Helias, 1962). The nature of the photoperiodic photoreceptor and its anatomical location will be considered further in Chapter 14.

ANNOTATED SUMMARY

1. Insects may show long- or short-day responses. Others show responses to changes from long to short days, from short to long days, or to naturally changing day lengths. Some species, on the other hand, are day-neutral. In the long-day response curve the most important physiological and ecological feature is the sharp discontinuity at the critical day length which separates the diapause-inducing short photoperiods from the long days which promote uninterrupted development.
2. Insects may also use temporal information from the daily *thermoperiod* to regulate diapause. Some species are able to distinguish short day (diapause inducing) thermoperiods from long day (diapause averting) thermoperiods in continuous darkness or, occasionally, in continuous light.
3. Insects are usually sensitive to photoperiod during a restricted part of their development. In some insects this sensitive period occurs in the same instar as the resulting diapause; in many more it precedes it. In the former case, diapause termination is frequently controlled by a photoperiodic mechanism (= oligopause), whereas in the latter it is more usually in response to a period of chilling (= eudiapause).
4. In a number of species the sensitive period occurs in the maternal generation, and the eggs may be 'determined' for subsequent diapause or non-diapause developmental pathways before they are deposited.
5. The photoperiodic response is variously modified by temperature. In most long-day species constant high temperature acts in accordance with long days to avert diapause, whereas low temperatures act in the same 'direction' as short days. In short-day or winter active species, high temperature may act to augment diapause, and lower temperature to avert it. Low- or high-temperature *pulses* may completely reverse the photoperiodic response.
6. Both the quality and the quantity of the diet may modify the photoperiodic response. Senescent vegetation or ripening fruit, for example, may act to intensify the short-day response in the autumn. In parasitic species, the species of host or its physiological 'condition' may also modify the response.
7. The photoperiodic response curve (PPRC) is a product of natural selection and differs in populations from different latitudes and geographical areas. At higher latitudes the longer summer days, the greater rate of change in day length, but shorter summer season, are reflected in a longer critical day length than in areas further south. More northerly populations frequently achieve fewer generations per year than those in the south, have a longer-lasting or more intense diapause and may show an increased cold hardiness.

Altitude and the proximity to the warming influence of oceans also affect the photoperiodic response and number of generations per year.

8. The important characters of the photoperiodic response (critical day length and diapause intensity, for example) are genetically controlled and therefore open to natural and artificial selection, and to experimental crosses between strains from different geographical areas. Artificial selection both for and against diapause incidence suggests that inheritance of photoperiodic and diapause traits is by a polygenic system.
9. Action spectra for the photoperiodic response indicate a variety of photopigments. Some insects show sensitivity not unlike that for the phase-shifting of circadian oscillations, with a sharp cut-off above 500 nm; others are distinctly red-sensitive. The pigments involved have not been identified, but evidence in favour of carotenoids has been obtained in Lepidoptera, aphids and mites. Intensity effects are generally related to habitat, and threshold sensitivities may be as low as 0.025 lux. Thresholds at dawn are usually lower than those at dusk.

This Page Intentionally Left Blank

CHAPTER 11

CIRCADIAN RHYTHMS IN PHOTOPERIODISM

I am a sundial, and I make a botch of what is done far better by a watch.
Hilaire Belloc

CONTENTS

Introduction	339
A. <i>Bünning's General Hypothesis</i>	340
B. <i>Evidence for the Involvement of the Circadian System in Photoperiodic Time Measurement.</i>	341
1. 'Skeleton' photoperiods and 'external coincidence'	341
2. Night interruptions in cycles with an extended 'night': the Bünsow protocol	344
3. The photoperiodic oscillation's phase response curve	348
4. Abnormal light-dark cycles	350
(a) Relative importance of the light and dark phases	350
(b) 'Resonance' experiments: the Nanda-Hamner protocol	351
(c) T-experiments	358
5. Symmetrical 'skeletons' and the 'bistability' phenomenon	360
C. <i>Evidence for Hourglass-like Timers in Photoperiodism</i>	364
1. Abnormal light dark cycles	364
2. Night interruption experiments	365
3. Symmetrical 'skeleton' photoperiods	367
4. The Veerman-Vaz Nunes protocol	368
D. <i>Species Showing 'Positive' Responses in one Condition, but 'Negative' in Another</i>	370
E. <i>Hourglass-like Clocks as Heavily Damped Circadian Oscillators</i>	371
Annotated Summary	374

INTRODUCTION

THE photoperiodic response curve describes the reaction of a population of a particular insect to different static photoperiods. Ecologically and physiologically the most important part of this curve is the sharp discontinuity between the long-days which allow continuous or diapause-free development and the short-days which induce diapause. This 'critical photoperiod' infers that the insect possesses some kind of inherited and intrinsic clock which enables it to 'measure' the length of the day or the night (or perhaps both) and respond with the appropriate seasonal switch in metabolism. As shown by the steepness of the curve at the critical point this form of time measurement is often performed with considerable accuracy.

Some species, for instance, are able to detect differences of as little as 10 or 15 minutes in day length, and a difference of one hour usually converts the response from one metabolic pathway to the other.

In earlier chapters we have discussed some of the seasonal events controlled by this mechanism, and the way in which the response is modified by certain environmental factors such as temperature, diet and latitude. In this chapter we turn our attention to the physiological mechanisms by which this time measurement is achieved. To date, rather little is known about the concrete physiological processes involved, but we have a considerable knowledge of the formal properties of the system. Attention will be directed almost entirely at the long-day response because this is the most common type in the insects, and practically all of the published work is concerned with this form of the response.

Photoperiodic induction is a two-stage process. The first involves the measurement of the duration of the dark or the light phases of the daily cycle by the clock. The second involves the summation of successive days' information on day length to a point at which induction occurs, or is irreversible; this process is accomplished by the 'photoperiodic counter', and will be the subject of Chapter 12.

A. BÜNNING'S GENERAL HYPOTHESIS

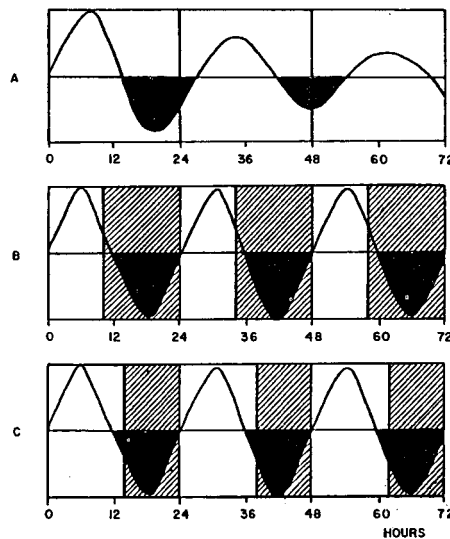


Fig. 11.1. Bünning's (1936) model for the execution of the photoperiodic time measurement by a circadian oscillation. A - the free-running clock in DD. B - under short days the light does not extend into the second or scotophil half-cycle, whereas in C (long days) it does. (After Bünning, 1960.)

Early workers were apparently not much concerned with the mechanism of time measurement in photoperiodism. Very often, however, the implication was that day- or night-length measurement (or sometimes both) was accomplished by means of an 'hourglass' type of mechanism which was set in motion at either dawn or dusk and proceeded at a (presumably) temperature-compensated rate. If - in the case of a dawn hourglass - dusk intervened before the

process had reached a certain stage the photoperiod was 'read' as a short day; if, on the other hand, the process reached completion before dusk it was 'read' as a long day. Dickson (1949), in his very comprehensive investigation of diapause induction in the oriental fruit moth *Grapholitha molesta*, found that induction was only fully expressed in cycles consisting of between 11 and 15 hours of darkness and 8 to 15 hours of light: he discussed both 'light' and 'dark reactions'. Way and Hopkins (1950) found that diapause in the tomato moth *Diataraxia oleracea* was averted in photoperiods of more than 15 hours, and supposed that "prolonged stimulation of the last instar larval brain by light falling on the photoreceptors induces formation of the adult growth hormone". Both of these theories imply a simple hourglass-like mechanism.

A totally different explanation, however, had been put forward in 1936 by the German botanist Erwin Bünning to explain similar phenomena in plants. His very original proposition was that photoperiodic time measurement was dependent on the 'endogenous diurnal rhythm' then known to provide temporal organisation in plants, such as the daily up-and-down leaf movements of the bean seedling, *Phaseolus*. Bünning (1936) proposed that the 24-hour period comprised two half-cycles differing in their sensitivity to light. The first 12 hours constituted a photophil or 'light-requiring' half-cycle and the second 12 hours a scotophil or 'dark-requiring' half-cycle. Short-day effects were thus produced when the light was restricted to the photophil, but long-day effects when it extended into the scotophil (Fig. 11.1). This model for the clock, therefore, comprised an *oscillator* as opposed to an hourglass, and focused attention on the many similarities between photoperiodism and the overt diurnal rhythms of behaviour and physiology.

Although this model received some attention, particularly in the German botanical literature of the 1950s, it was not considered as a serious alternative to the hourglass in insect photoperiodism until its introduction into that field by Bünning and Joerrens in 1960. Since then, this model, or variations of it, have dominated the field. Much of this chapter, therefore, is concerned with evidence for some kind of Bünning's general hypothesis. It will present the evidence for the involvement of circadian rhythmicity in photoperiodic time measurement. It will also discuss evidence for the alternative view, namely that photoperiodic time measurement (at least in some species) is carried out by a non-oscillatory or hourglass-like timer, and will end by presenting the working hypothesis that apparent hourglass-like clocks are merely heavily damped circadian oscillators. The rather large number of different clock models, many of them arising from Bünning's hypothesis, will be reviewed in Chapter 13.

B. EVIDENCE FOR THE INVOLVEMENT OF THE CIRCADIAN SYSTEM IN PHOTOPERIODIC TIME MEASUREMENT

1. 'Skeleton' photoperiods and 'external coincidence'

The earliest experiments specifically addressed to the problem of time measurement in photoperiodism were 'night interruption' experiments (or asymmetrical 'skeleton' photoperiods) in which the dark phase of an otherwise inductive cycle was systematically perturbed by a second, short light pulse, or 'light break'. Such 'night interruption' experiments have long been a useful experimental tool in the analysis of photoperiodic phenomena in plants and birds (Hardner and Bode, 1943; Bünning, 1960; Jenner and Engels, 1952; Kirkpatrick and Leopold, 1952). These experiments demonstrated that photoperiodic *reversals* could be obtained by light-breaks as short as a few minutes (Parker et al., 1946). The same technique

was later applied with varying results to arthropods, in particular to the oriental fruit moth *Grapholitha molesta* (Dickson 1949), the spider mite *Panonychus ulmi* (Lees, 1953b) and the pitcher plant midge *Metriocnemus knabi* (Paris and Jenner, 1959). The light-breaks were often placed centrally in the night and, unlike similar experiments with plants, found to have limited effect. Lees (1953b), for example, found that the dark component of an LD 8:16 cycle had to be interrupted by at least six additional hours of light when placed centrally (e.g. LD 8:5:6:5) before an appreciable reduction in the proportion of winter eggs was obtained. Photoperiodic reactivation of the diapausing larvae of the midge *Metriocnemus knabi*, on the other hand, was achieved with a centrally placed 1½-hour night interruption (e.g. with LD 10½:6:1½:6) (Paris and Jenner, 1959). When long-day effects were obtained they were interpreted as evidence that the length of the dark period of the cycle was more significant than the light, and that the 'light reaction' developed more slowly in arthropods than in plants (Lees, 1960b).

Systematic pulsing of the night as part of a test of Bünning's hypothesis was first carried out with insect material by Bünning and Joerrens (1960), using the cabbage butterfly *Pieris brassicae*. Two-hour supplementary light-pulses were placed at different positions in the long nights of diapause-inducing cycles (LD 6:18 and LD 12:12), and maximum inhibition of diapause was observed when the light-pulse fell between 14 and 16 hours after dawn (*Zeitgeber* time, Zt 14 to 16) in both instances. Thus the period of maximum sensitivity, considered to constitute the light sensitive 'scotophil', was found to occur during the early part of the night rather than in the middle.

Working with the pink boll worm moth, *Pectinophora gossypiella*, Adkisson (1964, 1966) extended this technique with the systematic interruption of a range of light-dark cycles (LD 6:18, LD 12:12 and LD 13:11) with 1-hour pulses. This procedure produced two discrete points of diapause inhibition, the first (point A) about 14 hours after lights-on (Zt 14) and the second (point B) about 14 hours before lights-off, in all cycles tested. Light-pulses placed in the middle of the night had little effect. Diapause inhibition was also obtained by Beck (1962a) in the European corn borer, *Ostrinia nubilalis*, using appropriately timed supplementary pulses of 30 minutes. In *Pieris rapae*, similar effects were noted with light pulses as short as 5 minutes (Barker, 1963), or even with pulses as short as those provided by an electronic photoflash with a discharge half-life of 0.0008 sec, provided that the supplementary pulses or flashes were correctly timed and of sufficient intensity (Barker et al., 1964). These studies showed that 'light reactions' in insects were just as rapid as those previously described in plants. Since these early papers, diapause inhibition in night interruption experiments has been obtained for a number of insect species. These include the green vetch aphid *Megoura viciae* (Lees, 1965, 1966a), the parasitic wasp *Nasonia vitripennis* (Saunders, 1967, 1968, 1969), the moths *Heliothis zea*, *H. virescens* (Benschoter, 1968) and *Adoxophyes reticulana* (Ankersmit 1968), the fruit fly *Drosophila phalerata* (Tyshchenko et al., 1972), the small cabbage white butterfly *Pieris rapae crucivora* (Kono, 1970), and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1975b). Most of these studies revealed two points of apparent light sensitivity, but considerable specific differences were also apparent. Since these differences affect the interpretation of the phenomenon they will be described in detail later in this section.

The demonstration of two periods of sensitivity to light in the dark component might have been difficult to interpret in terms of Bünning's general hypothesis which envisaged a single light-sensitive 'scotophil'. However, Pittendrigh and Minis (1964) noted the striking parallel between this technique (specifically Adkisson's results with *P. gossypiella*) and the use of asymmetrical skeleton photoperiods to entrain the pupal eclosion rhythm in *Drosophila pseudoobscura* (see Chapter 3). They suggested that the results were powerful, if circumstantial, evidence for the involvement of the circadian system in photoperiodic time

measurement, and proposed an explicit version of Bünning's general hypothesis to account for these data.

In its original form the 'External Coincidence' model (Pittendrigh and Minis, 1964) comprised a circadian oscillation of a hypothetical substrate (S) and a phytochrome-like pigment or enzyme (E). In the model the substrate oscillation was entrained by the whole of the photoperiod, *or* by the asymmetrical skeleton formed by the main photoperiod and the pulse, so that its highest concentration (S-max) fell in the night. The concentration of S above a certain threshold constituted the light-sensitive phase comparable to Bünning's 'scotophil'. The enzyme (E) was conceived to be in an active state (E_a) when the light was on, but in its inactive state (E_i) in the dark. A more modern version of the model showing its essential features is shown in Fig. 11.2 (see also Chapter 13).

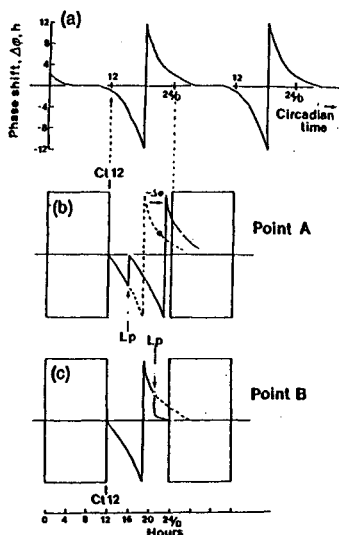


Fig. 11.2. The External Coincidence model for the photoperiodic clock and how it gives rise to two points (A and B) of long day effect. (a) the photoperiodic oscillator shown as a phase response curve. (b) under a long night regime (LD 12:12), a light pulse (L_p) falling early in the night causes a phase delay pushing the photoinducible phase (ϕ_i , closed circle) into the light, giving rise to point A. (c) conversely, a light pulse falling late in the night causes a phase advance until it coincides directly with ϕ_i giving rise to point B. From Pittendrigh and Minis. See also Figure 13.1.

In night-interruption experiments the oscillation achieved steady-state entrainment to the asymmetrical skeleton photoperiod formed from the main light period and the pulse. When the pulse fell early in the night it was 'read' as a terminator, or *new dusk*; when it fell late in the night it was 'read' as an initiator, or *new dawn*. Between these two points it could be accepted as neither, and a phase-jump (ψ -jump) occurred. Very similar behaviour was observed in the entrainment of the pupal eclosion rhythms of *D. pseudoobscura* and *P. gossypiella* to similar skeletons (see Chapter 3). In terms of photoperiodic induction the model predicted diapause-avoidance, or long-day effects, at two points. One was when the pulse acted as a terminator and coincided with S-max early in the night; the other was after the ψ -jump when the pulse, now acting as an initiator, phase-set the oscillator so that S-max coincided with the end of the main photoperiod.

Since there was no concrete evidence for a substrate oscillation as such, and still less for a phytochrome-like pigment in animals, the final form of the coincidence model (Pittendrigh, 1966) was reduced to a light-sensitive oscillation in which a *photoperiodically inducible phase* (ϕ_i) assumed a definite phase-relationship to the light-cycle when in steady state. The important details of the model, such as the mechanism of entrainment and the ψ -jump, however, were retained.

Although two points of 'light-sensitivity' were produced in response to light breaks, Pittendrigh (1966) pointed out that only one could represent the position of ϕ_i : after the ψ -jump ϕ_i was probably a full half-cycle away from its first position. However, since data for the eclosion rhythm in *D. pseudoobscura* (see Chapter 3) quite clearly demonstrated that the driving oscillation was 'damped out' or reset by light periods in excess of about 12 hours, in such a way that the onset of darkness always corresponded to Ct 12, he suggested that any extension of the main light component above 12 hours would effectively cause 'dawn' to move backwards into the preceding night (see Fig. 13.1). On the basis of this argument, Pittendrigh (1966) suggested that ϕ_i lay *late* in the night (at B) rather than at A. Stronger, and experimental, evidence for this contention will be provided later for *Sarcophaga argyrostoma* (Chapter 13). The reason why diapause-inhibition was greater at B than at A, however, was not explained, although the photoreceptors might be fully dark-adapted after a prolonged period of darkness.

The interpretation of 'night interruption' experiments in terms of the external coincidence model is in itself evidence for the involvement of the circadian system in photoperiodic time measurement. The model is particularly successful because it draws attention to three important points: (1) the proposed 'scotophil' or photoinducible phase (ϕ_i) is restricted to a much shorter light-sensitive phase than Bünning's entire 12-hour half-cycle; (2) light has a *dual role*, serving to entrain the oscillation and to effect induction by the temporal coincidence of light and ϕ_i ; and (3) it draws a close parallel between the photoperiodic clock and the known properties of circadian rhythms, as outlined in Chapters 2 and 3. The external coincidence model will be critically examined in Chapter 13.

2. Night interruptions in cycles with an extended 'night': the Bünsow protocol

'Night interruptions' in a 24 hour light-dark cycle (see above) led to the development of the external coincidence model for photoperiodic time measurement (PPTM) (Pittendrigh and Minis, 1964; Pittendrigh, 1966). They were therefore strong, if circumstantial, evidence in favour of an explicit form of Bünning's general hypothesis and, consequently, for a role for the circadian system in PPTM. Unequivocal evidence for such an association, however, could only come from experiments in which the cycle length (T) was extended beyond 24 hours. In particular, two experimental designs have been widely used: the Bünsow and the Nanda-Hamner protocols (see Fig. 11.3). The Bünsow type of experiment will be described here; the Nanda-Hamner or 'resonance' experiment in Section B4.

In the Bünsow type of experiment, the 'nights' of extended light-dark cycles (i.e. T = 48 or 72 hours) are systematically interrupted by additional short light pulses, generally of 1 or 2 hours. The results of such experiments frequently show periodic maxima of long- or short-day effects recurring with a circadian frequency in the extended night. In the long-day plant *Hyoscyamus niger*, for example, 2-hour light breaks placed systematically in the dark phase of a 48 hour cycle (LD 9:39), revealed maxima of flower induction at *Zeitgeber* time (Zt) 16 and Zt 40, 24 hours apart (Claes and Lang, 1947). In the *Biloxi* variety of soybean maintained in a light-dark cycle of LD 8:64 (T = 72 hours), flowering was induced when the scanning light

pulses fell at Zt 16, Zt 40 and Zt 64 (K. C. Hamner, 1960). Similar results were obtained for the plant *Kalanchoë blossfeldiana* in 48-hour (bidiurnal) and 72-hour (tridiurnal) cycles (Bünsow, 1953; Melchers, 1956; Bübbing, 1960) and, in animals, for testicular growth in the house finch *Carpodacus mexicanus* (W.M. Hamner, 1964).

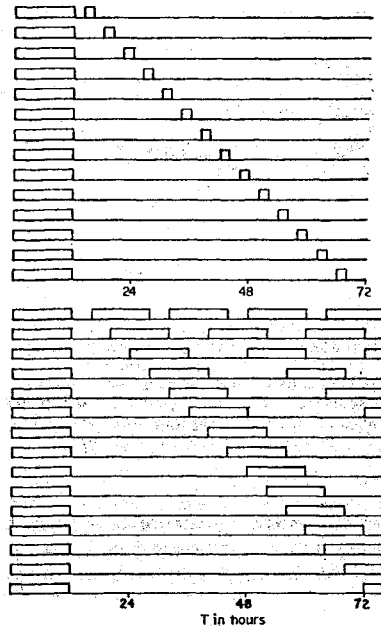


Fig. 11.3. Experimental designs for investigating photoperiodic phenomena. Upper panel: The Bünsow protocol: a long cycle ($T = 72$ hours) with a short 'main' photophase (12 hours in this case) and a scanning pulse. Lower panel: The Nanda-Hamner protocol: a series of cycles, each with a short 'main' photophase (12 hours in this case), but with the period of the cycle (T) varying over wide limits (from, say, $T = 16$ to $T = 72$ hours). With both approaches, the organism is subjected to the experimental light-cycles throughout its sensitive period.

Similar experiments have now been performed with a number of insect species and produced a variety of responses (Table 11.1). These responses fall into two broad categories, dubbed 'positive' and 'negative' Bünsow effects. 'Positive' effects, like those reviewed above for plants and the sparrow, show repeated points of long day effect (diapause avoidance) as the supplementary light pulse scans the night. The most persuasive of these experiments are those using tridiurnal cycles where $T \sim 3\tau$ hours, and three successive points of diapause avoidance are obtained. In insects, however, 'negative' responses have also been recorded, most notably for the aphid *Megoura viciae* (Lees, 1973), long considered the prime example of a non-circadian or 'hourglass-like' timer (see below). Some of these results, 'positive' and 'negative', will be considered here in more detail.

Among the insects, 'positive' Bünsow responses were first recorded in the parasitic wasp, *Nasonia vitripennis* (Saunders, 1970). Females of the wasp were maintained in 48-hour and 72-hour cycles containing a short 'main' photoperiod of between 10 and 14 hours and a supplementary pulse of 2 hours. The wasps were supplied with two fresh puparia of its flesh fly host daily, and the type of progeny (diapause or non-diapause) scored for each day of the

experiment. In Fig. 11.4 short day effects (a high incidence of diapause) were indicated by a rapid switch to the production of diapausing progeny, whereas long-day effects were indicated by a delayed switch. In a cycle of LD 12:36 ($T = 48$ hours), long-day effects, or points of apparent sensitivity to light, were observed when the pulses fell at Zt 19 and Zt 43 - 24 hours apart. Similar experiments with LD 10:38 and LD 14:34 produced peaks in the same positions indicating that time measurement began with dawn rather than dusk. In a 72-hour cycle (LD 12:60) three such peaks were produced, one at Zt 19, one at Zt 43 and a third at Zt 67 - once again, 24 hours apart. These results indicated that points of sensitivity to light recurred with circadian frequency in the extended night. The central point was particularly informative because it was unlikely to arise from a direct interaction between the pulse and the 'main' photoperiod.

TABLE 11.1. Bünsow experiments; table modified from Vaz Nunes and Saunders (1999).

Species	Response	Temperature	Duration of light pulses	Reference
<hr/>				
'Positive' circadian effects; T = 3τ				
<i>Nasonia vitripennis</i>	Diapause induction	18°C	2 hours	Saunders (1970)
<i>Ostrinia nubilalis</i>	Diapause induction	23°C	2 hours	Bonnemaïson (1978)
<i>Sarcophaga argyrostoma</i>	Diapause induction	16 - 17°C	1 hour	Saunders (1976)
<i>Tetranychus urticae</i>	Diapause induction	19°C	1 hour	Vaz Nunes & Veerman (1984)
<i>Dianemobius fasciatus</i>	Wing form	25 - 28°C	1 hour	Masaki et al. (1992)
<i>D. nigrofasciatus</i>	Wing form, egg diap. & larval development			
'Positive' circadian effects; T = 2τ				
<i>Aedes atropalpus</i>	Diapause induction	22°C	1 hour	Beach & Craig (1977)
<i>Pieris brassicae</i>	Diapause induction	20°C	1 hour	Dumortier & Brunnari- us (1981)
<i>Adoxophyes orana</i>	Diapause induction	23°C	2 hours	Bonnemaïson (1978)
<i>Aphis fabae</i>	Apterisation	20°C	1 hour	Hardie (1987b)
'Negative' circadian effects				
<i>Megoura viciae</i>	Ovipara production	15°C	1 hour	Lees (1973)
<i>Plodia interpunctella</i>	Diapause termination	21°C	1 hour	Takeda & Masaki (1976)
<i>Aphis fabae</i>	Apterisation	15°C	1 hour	Hardie (1987b)
<i>Drosophila triauraria</i>	Diapause induction	15°C	1 hour	Yoshida & Kimura (1993)
<i>Mamestra brassicae</i>	Diapause induction	20°C	1 hour	Kimura & Masaki (1993)

A similar experiment was performed with larvae of the flesh fly *Sarcophaga argyrostoma* maintained in light cycles (T = 72 hours) each containing a 12-hour 'main' photoperiod, a 60-hour extended dark period, and a 1-hour scanning pulse (Saunders, 1976). The results of this experiment (Fig. 11.5) were consistent with the view that diapause induction in *S. argyrostoma* was also a function of the circadian system. Thus, 1-hour pulses of light placed within each of the subjective days (Zt 24-36, and Zt 48-60) produced a high level of diapause (or short-day effect). In contrast, similar pulses placed within each of the three subjective nights (Zt 12-24, Zt 36-48, and Zt 60-72) resulted in a lowered incidence of diapause (long-day effect). The bimodality of diapause reversal observed in experiments of this kind using T = 24 hour cycles (see Section B.1), and apparently missing in *N. vitripennis* (Fig. 11.4), may be preserved in *Sarcophaga* with two points of 'sensitivity' (one at about Zt 38 and the other at Zt 46) being discernible in the second subjective night. The second of these points may represent the position of the photoinducible phase (ϕ_i).

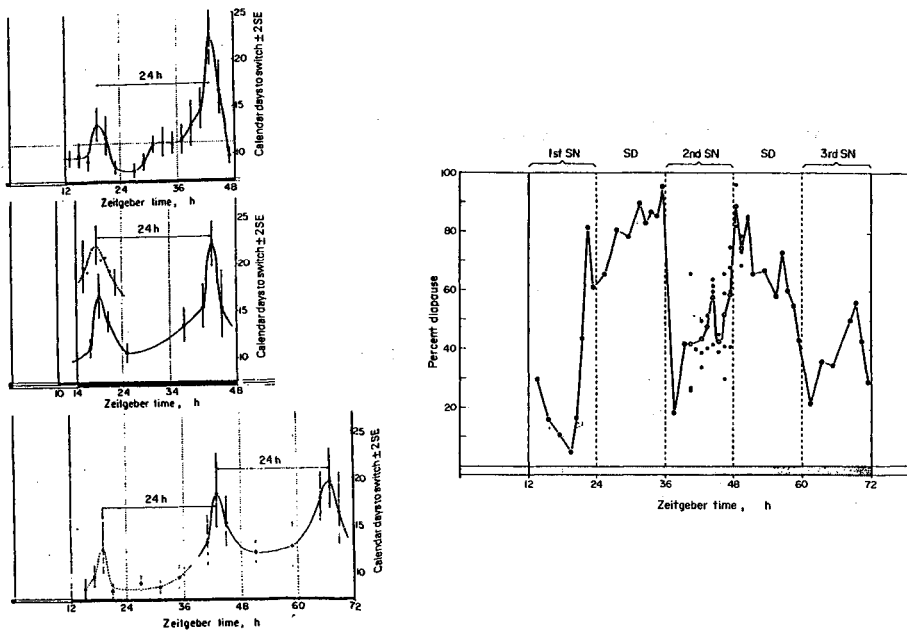


Fig. 11.4 (left). 'Positive' Bünsow experiments with the parasitic wasp, *Nasonia vitripennis*. Top panel: long day effects of 2 hour light breaks in an LD 12:36 cycle (T = 48 hours). Middle panel: effects of 1 hour light breaks in LD 13:34 (dashed line) and 2 hour light breaks in LD 10:38 (solid line) (T = 48 hours). Lower panel: effects of 2 hour light breaks in a cycle of LD 12:60 (T = 72 hours). Peaks of long-day effect (diapause inhibition) are produced at 24 hour intervals in the extended night with the first peak always 19 hours after dawn. Each point represents the mean age at which the female wasps 'switch' from the production of nondiapausing to diapausing offspring (n = 40).

Fig. 11.5 (right). 'Positive' Bünsow experiment with the flesh fly *Sarcophaga argyrostoma*. 1-hour supplementary light-pulses systematically scanning the extended 'night' of an LD 12:60 cycle (T = 72 hours). SN - subjective nights (Zt 12-24, 36-48, 60-72); SD - subjective days (Zt 24-36, 48-60). Closed circles - proportion of larvae entering pupal diapause in particular interrupted regime; open circles - mean values of the same. Diapause inhibition occurs when supplementary light pulses fall in each of the subjective nights (From Saunders, 1976).

'Positive' Bünsow effects have now been observed in a number of other species of insect and a mite; these are listed in Table 11.1. In the mosquito *Aedes atropalpus*, observations were confined to cycles of $T = 48$ hours (LD 10:38) with a 1 hour scanning pulse (Beach and Craig, 1977). Points of 'sensitivity' to the light break (lowered diapause) were observed at Zt 16-18 and, 24 hours later, at Zt 40-42. Similarly, in the spider mite, *Tetranychus ulmi*, in LD 12:52 ($T = 64$ hours) three minima of diapause incidence were observed (Vaz Nunes and Veerman, 1984). Here the interval between them was only 20 hours.

On the other hand, 'negative' Bünsow effects have also been recorded (Table 11.1). Apart from *Megoura viciae* (Lees, 1973) already mentioned, circadian peaks or troughs in photoperiodic effect were not observed in the Indian meal moth *Plodia interpunctella* (Takeda and Masaki, 1976), the black bean aphid *Aphis fabae* (Hardie, 1987), the fruit fly *Drosophila triauraria* (Yoshida and Kimura, 1993) or in the cabbage moth *Mamestra brassicae* (Kimura and Masaki, 1993). However, in several of these species, 'positive' (i.e. circadian) responses were observed in Nanda-Hamner or 'bistability' experiments (see below). These apparent anomalies will be addressed later (Section D).

3. The photoperiodic oscillator's phase response curve

If photoperiodic induction is a function of the circadian system, the photoperiodic oscillation must possess a phase response curve (see Chapter 3, C. 1). The demonstration of such a PRC is, however, difficult because of the covert nature of the photoperiodic oscillation, and has been attempted only twice, once with the plant *Chenopodium rubrum* (King, in Cumming, 1971) and once in *Sarcophaga argyrostoma* (Saunders, 1976).

In *S. argyrostoma* the PRC was obtained by the use of 3-point skeleton regimes ($T = 72$ hours). Each light cycle was formed from a 'main' light component of 12 hours (P_1), a 15-minute 'resetting' pulse (P_2) timed to fall at different times after the end of P_1 and, for each combination of P_1 and P_2 , a series of 1-hour 'scanning' pulses (P_3) timed to occur during the second subjective night to determine the diapause response (Fig. 11.6A). The rationale behind this procedure was as follows. In each 72-hour cycle, the 12-hour main photoperiod (P_1) was assumed to act as a 'strong' resetting stimulus (Winfree, 1970; Chapter 3, C. 1) and therefore resetting the oscillation to a near constant phase regardless of the phase of the system at its beginning. The second 15-minute light-pulse (P_2) was then assumed to cause first phase delays ($-\Delta\phi$) and then phase advances ($+\Delta\phi$) as it scanned the first subjective night. The scanning pulses (P_3) were placed at different times in the *second* subjective night to detect changes in the position of the second point of long-day effect (Fig. 11.6 B and C). These complex three-point skeletons were repeated throughout the insects' sensitive period, from larviposition to puparium-formation.

A total of 96 separate cultures, each of 500 to 700 larvae, were used in this experiment. Two results, one for P_2 at Zt 15 (early subjective night) and one for P_2 at Zt 18 (late subjective night), are shown in Fig. 11.6 B and C, where they are compared with the diapause response, in the same region, for a 2-point skeleton, taken from Fig. 11.5. When P_2 fell in the early subjective night it caused a phase delay of about 2 hours, whereas in the *late* subjective night it caused an *advance* of about 2 hours. Figure 11.6D shows all such experiments plotted as a function of the time after transfer from the 12-hour photoperiod (P_1) into darkness, and compares the results with similar phase changes for the rhythm of pupal eclosion. Both sets of data provide a PRC of Winfree's 'weak' Type I (see Chapter 3, C. 1) with delays of up to 2 hours and advances up to 3 hours.

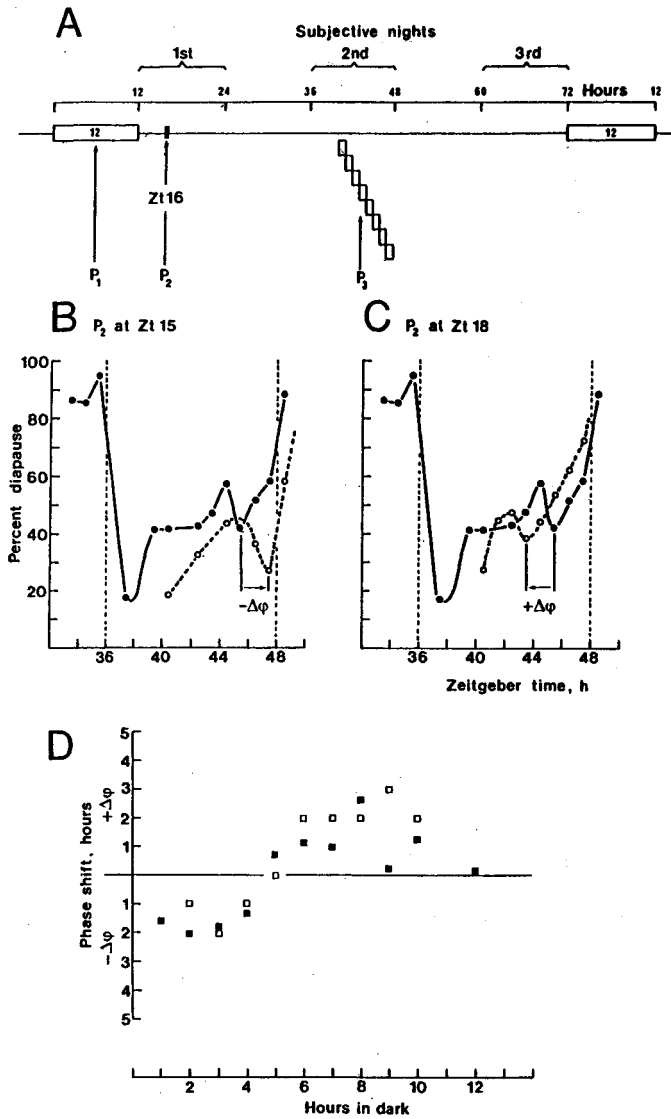


Fig. 11.6. *Sarcophaga argyrostoma*, phase response curve (PRC) for the photoperiodic oscillator. A – format of the 3-point skeleton photoperiods used (P_1 – the main 12 hour light period; P_2 – the 15 minute resetting pulse; P_3 – the 1 hour scanning pulses). B and C – phase changes elicited by resetting pulses falling at Zt 15 and Zt 18. $-\Delta\phi$ delay phase shifts; $+\Delta\phi$ advance phase shifts. D – open squares, PRC for the photoperiodic oscillator (15 minute pulses); closed squares, PRC for the rhythm of pupal eclosion for comparison (1 hour light pulses following 3 days at LD 12:12) (From Saunders, 1976).

4. Abnormal light-dark cycles

The second main approach to the problem of time measurement has been the study of photoperiodic induction in light-dark cycles in which the period (T) is not equal to τ and the hours of light and dark are varied independently of each other. Most of the earlier experiments of this type were confined to cycles in which T was close to τ , and were designed to determine whether the light component was 'more important' than the dark, or vice versa. Later experiments - including T-experiments and Nanda-Hamner or resonance experiments (see Fig. 11.3) - consisted of much longer cycles (up to 72 hours or more) containing a short light component and a protracted night.

(a) Relative importance of the light and dark phases

Experiments in which the light and the dark components of the cycle are independently varied have frequently shown that the length of the 'night' is more critical than the length of the 'day'. Consequently many earlier investigators considered that night length was of central importance in time measurement. In some species, particularly the green vetch aphid *Megoura viciae*, night length measurement was performed with little reference to the length of the accompanying light period, and the 'clock' was interpreted as a dark-period interval timer or hourglass (Lees, 1966, 1968) (see Section C). In most species, however, both light and dark periods were shown to require a definite duration if induction (of diapause) is to occur.

Dickson (1949) investigated the induction of larval diapause in the Oriental fruit moth *Grapholitha molesta* in a wide range of abnormal cycles. In cycles where T varied from 18 to 30 hours, diapause was only induced when the dark component (D) was in excess of 10 to 11 hours, but less than 16 hours. Holding D constant at 11 hours he then found that the light period (L) had to be between 8 and 15 hours. The strongest diapause-inducing effects were noted when D and L between these limits added up to about 24 hours. Since the range for D was smaller than that for L he concluded that dark-period measurement was more important. Abnormal cycles in which the ratio of D to L was held at 1:1 were less informative, but showed that diapause was only induced when cycle duration (T) was between 20 and 26 hours.

Similar results indicating the importance of the dark period were obtained for other insect species. In the knot grass moth *Acronycta rumicis*, for example, the dark period had to be in excess of 9 hours (Danilevskii and Glinyanaya, 1949). And almost all of the pupae of the silk moth *Antheraea pernyi* entered diapause when D was greater than 11 hours, even if L was as long as 59 hours (Tanaka, 1950, 1951). In *Pieris rapae*, pupal diapause was induced only if D was greater than 12 hours in cycles where $T = 20$ to 36 hours; within these limits the length of the light was much less important (Barker and Cohen, 1965). The central importance of night length was also seen in data for the flesh fly *Sarcophaga argyrostoma* (Saunders, 1973b). The incidence of pupal diapause was found to be very low in cycles containing a *short* night (LD 12:8 and LD 16:8) but approached 100 per cent in cycles containing a *long* night (LD 12:12 and LD 16:12), regardless of whether the accompanying light component was 'short' or 'long'.

Working with the European corn borer *Ostrinia nubilalis*, Beck (1962a) studied the effects of 10, 12 and 14 hours of light in conjunction with a wide range of dark periods. Maximum incidence of diapause (90 per cent and over) occurred with dark periods of 10 to 14 hours. Dark periods of this length, however, produced a high incidence of diapause when combined with a wider range of light (5 to 18 hours). Once again maximum induction occurred when $D + L$ was close to 24 hours. This relationship was well illustrated in the 'isoinduction

surface' calculated by Pittendrigh (1966, 1972) from Beck's data (Fig. 11.7). Tyshchenko et al. (1972) compared diapause induction and entrainment of the oviposition rhythm of *Drosophila phalerata* in a series of cycles between $T = 20$ and $T = 34$ hours in which $L = D$. The range of effective cycles was similar in both cases.

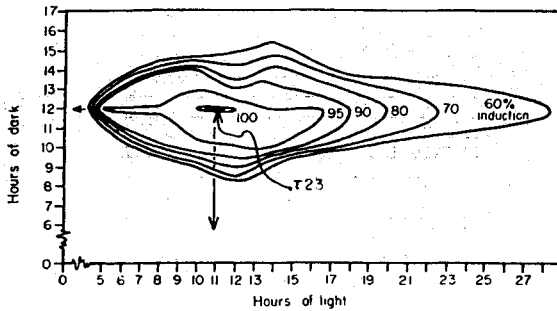


Fig. 11.7. *Ostrinia nubilalis*. A surface of 'isoinduction' lines, based on data given by Beck (1962). The lines connect combinations of light and darkness that give rise to equal levels of diapause. The surface shows the dominant role of the dark phase (maximum about 12 hours) peaking close to 11 hours of light to give maximum induction at LD 11:12 ($T = 23$ hours) (From Pittendrigh, 1966).

These data show that dark-period measurement frequently occupies a 'central' role in photoperiodism, although the duration of dark *and* light are both important, and the most inductive cycles are often those in which $L + D$ are close to 24 hours. Dickson (1949) suggested that time measurement in *G. molesta* involved two reactions, one requiring dark and the other requiring light. Beck (1962a) was inclined to interpret his data for *O. nubilalis* in terms of an 'interval timer'. However, the over-riding importance of a cycle length of $T \sim 24$ hours in this species (and many others), led Beck to interpret the results as "lending at least feeble support to the hypothesis that photoperiodic induction of diapause involves a circadian function" (see also Beck, 1988). Later in this chapter we will see that dark-period measurement may only become important in certain cycles.

Although most species illustrate the central importance of night length in photoperiodic time measurement, there are some species that seem to show the opposite. One such example is the linden bug, *Pyrrhocoris apterus* (Saunders, 1987b). In this species, independent variation of the dark ($D = 7, 8, 9$ or 12 hours) and light ($L = 12, 15, 16$ or 17 hours) components of the cycle showed that a 'critical day length' of about $15\frac{1}{4}$ hours/24 was more important than the reciprocal $8\frac{1}{4}$ hours of darkness. In most insects, however, as reviewed above, night length is of central importance.

(a) 'Resonance' experiments: the Nanda-Hamner protocol

A logical extension of the type of experiment described above is to hold the light-period constant (at, say, 6 to 12 hours) and to vary the dark component over a very wide range to provide environmental light cycles (T) up to 72 hours or more in length. This experimental design (Fig. 11.3) was first used for plants by K. C. Hamner and his associates (Blaney and Hamner, 1957; Hamner, 1960; Takimoto and Hamner, 1964), and later for birds (W. M. Hamner, 1963), and has become known as the Nanda-Hamner protocol. Results for plants and

birds showed periodic maxima of induction when T was close to 24, 48 or 72 hours, but minima of induction when T was close to 36 or 60 hours. The interpretation of such 'resonance' experiments is as follows. If the photoperiodic clock incorporates a circadian oscillation (i.e. with an endogenous periodicity, τ , close to 24 hours), the product of induction (e.g. flower induction, testis growth, or diapause) is observed to be high when T is close to τ or modulo τ , but *low* when T is far from τ . In the former, the endogenous oscillation and the light cycle 'resonate'; in the latter, they do not. This type of experiment has now been applied to about 23 species of insects and mites, 12 of which show 'positive' or rhythmic responses, 8 apparently do not, and three exhibit 'positive' or 'negative' responses in different studies (Tables 11.2 and 11.3).

TABLE 11.2. Nanda-Hamner experiments determined at one temperature only. Table modified from Vaz Nunes and Saunders (1999).

Species	Response	Temperature, τ h	Reference
'Positive' resonance effects			
<i>Nasonia vitripennis</i>	Diapause induction	18°C; τ = 24 h	Saunders (1968, 1974)
<i>Pterostichus nigrita</i> , German strain, 51°N	Diapause induction	15 - 20°C; τ = 24 h	Thiele (1977)
<i>Pieris brassicae</i> , French strain	Diapause induction	20°C; τ = 21 h	Dumortier and Brunnarius (1981)
<i>Pteronemobius fascipes</i>	Larval development	25°C; τ = 24 h	Takeda (1986)
	Diapause termination	25 - 28°C; τ = 24 h	Masaki et al. (1992)
<i>Dianemobius fascipes</i>	Wing form	25 - 28°C; τ = 24 h	Masaki et al. (1992)
<i>Tetranychus urticae</i> Dutch strain, 52°N	Diapause induction	19°C; τ = 19.5 h	Vaz Nunes & Veerman (1986a)
Various strains 40.5-60°N	Diapause induction	19°C; τ = 17.75-21.5	Vaz Nunes et al. (1990b)
Russian strain, 60°N	Diapause termination	19°C; τ = 16 h	Veerman & Koveos (1989)
<i>Drosophila melanogaster</i> Canton-S	Diapause induction	12°C; τ ~ 24 h	Saunders (1990)
'Negative' resonance effects			
<i>Metatetranychus ulmi</i>	Diapause induction	15°C	Lees (1953b)
<i>Pectinophora gossypiella</i>	Diapause induction		Pittendrigh & Minis (1971)
<i>Plodia interpunctella</i>	Diapause termination	21°C	Takeda & Masaki (1976)
<i>Pterostichus nigrita</i> Lapland strain, 64-66°N	Diapause induction		Thiele (1977b)
<i>Pieris brassicae</i>	Diapause termination	20°C	Claret (1985)
<i>Adoxophyes orana</i>	Diapause induction	23°C	Bonnemaïson (1977)
<i>Acyrtosiphon pisum</i>	Ovipara production		Lees (1990)
	Male production		
<i>Pimpla instigator</i>	Diapause termination		Claret & Arpagaus (1994)

TABLE 11.3. Nanda-Hamner experiments determined at more than one temperature. Table modified from Vaz Nunes and Saunders (1999).

Species	Response	Temperatures	Reference
'Negative' resonance at all temp. tested			
<i>Aleyrodes proletella</i>	Diapause induction	15, 17 and 20°C	Adams (1986b)
<i>Megoura viciae</i>	Ovipara production	15, 18 and 20°C	Lees (1986)
<i>Ostrinia nubilalis</i>	Diapause termination	20 and 30°C	Skopik and Takeda (1986)
'Positive' and 'negative' resonance dependent on temp.			
<i>Sarcophaga argyrostoma</i>	Diapause induction	14 to 28°C; negative at low temperature	Saunders (1973b; 1982c)
<i>Drosophila auraria</i>	Diapause induction	15°C no resonance; 17°C $\tau = 24$ h	Pittendrigh (1981)
<i>Dendroides canadensis</i>	Antifreeze protein	17 and 20°C $\tau = 24$ h	Horwath & Duman (1984)
<i>Ostrinia nubilalis</i> three strains	Diapause induction	20 and 30°C, resonance variable	Takeda & Skopik (1985)
Wisconsin strain	Diapause induction	19 - 25°C $\tau = 24$ h	Beck (1988)
<i>Aphis fabae</i>	Apterisation	30°C no resonance; 15°C no resonance; 20°C $\tau = 20$ h	Hardie (1987b)
<i>Pieris brassicae</i>	Diapause induction	19°C no resonance; 22.5°C $\tau = 20-24$ h	Veerman et al. (1988)
<i>Amblyseius potentillae</i>	Diapause induction	19°C no resonance; 22.5°C $\tau = 20-24$ h	Van Houten & Veenendaal (1990)
<i>Calliphora vicina</i>	Diapause induction	20°C no resonance; 23.5°C $\tau = 24$ h	Vaz Nunes et al. (1990)
<i>Drosophila triauraria</i>	Diapause induction	15°C no resonance; 17°C $\tau = 24$ h	Yoshida & Kimura (1993)
<i>Mamestra brassicae</i>	Diapause induction	20°C very small trough at T = 36 h; 23-25°C $\tau = 24$ h; 28°C very little diapause	Kimura & Masaki (1993)

'Positive' Nanda-Hamner responses were first demonstrated, in insects, for the parasitic wasp, *Nasonia vitripennis* (Saunders, 1968, 1974). Female wasps were exposed for 19 days to different environmental light cycles in which the light phases ranged from 4 to 28 hours, and the period of the cycle (T) from 12 to 72 hours, different experimental groups receiving repeated cycles of a different duration. The wasps were supplied throughout the experiment with puparia of their flesh fly host. Between the 19th and 21st days of the experiment the surviving females were separated and provided with two fresh hosts, and the type of progeny produced during this period (diapause or nondiapause) assessed by incubating the parasitised

puparia for a further 10 days. For each light cycle the results were calculated as the percentage of wasps producing diapausing progeny during the test period.

When a 12-hour photophase was employed, maxima of diapause induction were observed when the wasps were exposed to cycles of $T = 24$ to 28 hours, and at $T = 48$ to 52 hours; a third peak was also evident at about $T = 72$ hours. Minima of diapause incidence were observed at $T = 36$ and 60 hours (Fig. 11.8). When the photophase was increased from 12 hours to 14 and then to 16 hours the 'descending slopes' of the peaks remained in the same positions (at $T = 28$ to 32, and at $T = 52$ to 56 hours). The 'ascending slopes', however, moved to longer T values, in such a way that the peaks became narrower. When a 20-hour photophase was employed diapause was practically eliminated. A similar but opposite trend was observed when the photophase was shortened to 8 and 4 hours: the ascending slopes moved steadily to the left whereas the descending slopes remained at the same point, except for the 4-hour photophase where a perceptible movement to the left was also apparent.

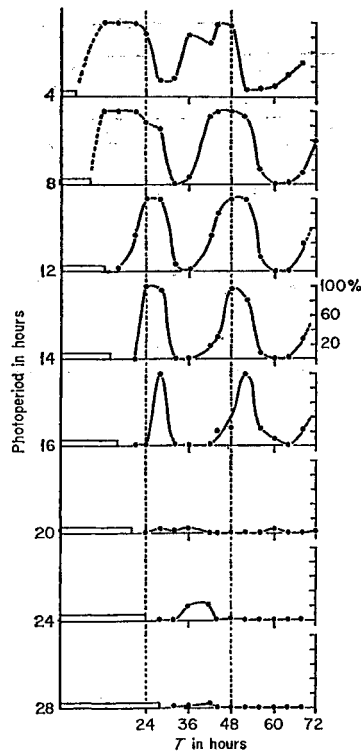


Fig. 11.8. 'Positive' Nanda-Hamner experiment with the parasitic wasp, *Nasonia vitripennis*. Production of diapausing larvae by female wasps maintained at 17°C in cycles up to 72 hours in length, containing photophases of 4 to 28 hours; periodic maxima occur about 24 hours apart. As the photophase increases from 4 to 16 hours the 'ascending slopes' of the diapause maxima move to the right (i.e. follow dusk) whereas the 'descending slopes' retain a fixed relationship to dawn. With a 20 hour photophase diapause is eliminated at all T values, but reappears in a peak at T 36 to 42 hours with a 24 hour photophase. The 'ascending' and 'descending' slopes may be interpreted as the manifestations of a dawn and dusk oscillator, respectively (From Saunders, 1974).

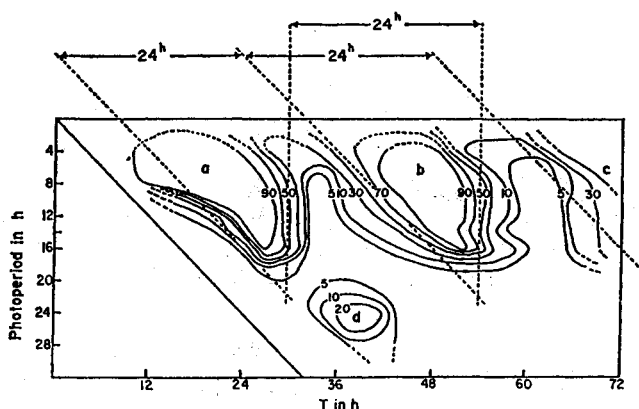


Fig. 11.9. Data from Fig. 11.8 redrawn as a 'circadian topography' in which all points of equal diapause induction are joined by contours. This figure shows the relationship of the putative 'dawn' and 'dusk' oscillators to the photophase which is drawn on the left as a 'light wedge' (From Saunders, 1974).

Figure 11.9 shows the same data replotted as a 'circadian topography' in which all points of equal diapause incidence are joined by 'contours' to give an 'isoinduction surface' (Pittendrigh, 1965, 1972). This figure clearly shows the ascending and descending slopes of the diapause maxima, and their relationship to the photophase which is shown at the left of the diagram as a 'light wedge'. It is clear that the ascending slopes are parallel to dusk and the descending slopes are parallel to dawn; both repeat themselves in the extended dark period with circadian (~ 24 hour) frequency. The two slopes may be interpreted as reflecting two independent components in the photoperiodic clock, one obtaining its time cue from dawn and the other from dusk. Since they reset themselves with a 24-hour frequency they are both regarded as circadian oscillators, rather than hourglasses that would not recur in such a manner. With a 20-hour photophase the two components appear to coalesce and diapause is eliminated at all T-values. On the other hand, with a 4-hour photophase the two components appear to diverge so that the central maximum of diapause induction divides into two distinct peaks. Finally when a 24-hour photophase was used an additional, discrete, peak of diapause induction (peak d) appeared at $T = 36$ to 42 hours. This suggests that the two oscillators enter a 'forbidden' mutual phase-relationship and one - probably the dawn oscillator - then executes a $180^\circ \psi$ -jump, thereafter continuing to function as the dawn component. The fact that an additional peak appears with very long light periods (>24 hours) suggests that the dawn oscillator is not damped out by protracted illumination, as is the oscillator controlling adult eclosion in *D. pseudoobscura* (Pittendrigh, 1966) (Chapter 3). For further interpretation of these Nanda-Hamner results in *N. vitripennis*, see Chapter 13.

Nanda-Hamner experiments conducted with the flesh fly *Sarcophaga argyrostoma* have also shown that photoperiodic induction is a function of the circadian system, although significant differences exist between this species and *N. vitripennis* (Saunders, 1973b, 1982c). Larvae of *S. argyrostoma* were raised at 17°C and in a range of light-cycles from $T = 20$ to $T = 72$ hours, each cycle containing from 4 to 20 hours of light. Newly formed puparia were collected daily from these cultures, either during the light component of the cycle, or under red light (>600 nm) which they apparently do not 'see' (Chapter 14). The puparia were opened about two weeks later to ascertain the diapause or nondiapause status of the intrapuparial stages. The results showed periodic maxima of diapause induction, but the positions of the peaks varied according to the duration of the photophase (Fig. 11.10). The results are also pres-

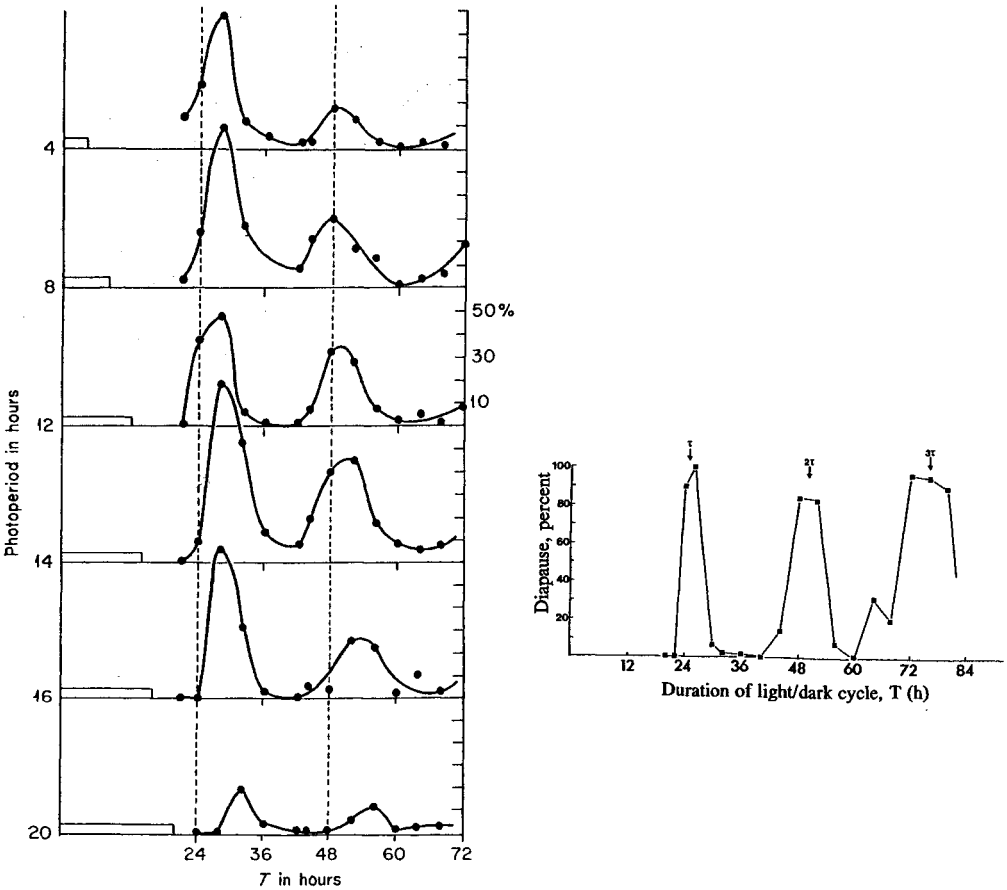


Fig. 11.10. 'Positive' Nanda-Hamner experiments with (Left) the flesh fly, *Sarcophaga argyrostoma* and (Right) the blow fly, *Calliphora vicina*. At left - the incidence of pupal diapause in *S. argyrostoma* when the larvae were reared at 17°C in cycles up to 72 hours containing photophases of 4 to 20 hours; periodic maxima of diapause occur at about 24 hour intervals (After Saunders, 1973b). On the right - similar data for *C. vicina* in experiments at 23°C with a 13 hour photophase (From Saunders, 1998).

ented in the form of a 'circadian topography' (Fig. 11.11) to illustrate the temporal relationship of the diapause maxima to the photophase, which is drawn in the figure as a 'light wedge'. This shows that the second peak reached its maximum at $T = 48$ hours for a 4-, 8- or 12-hour photophase, but 'moved' to the right by 2, 4 and 8 hours, respectively, for the 14-, 16- and 20-hour photophases. This suggests that the circadian system, or that part of the circadian system associated with photoperiodism, attains a phase-relationship to the light cycle in such a way that it receives its principal time cue from dusk once the photophase exceeds about 11 to 12 hours. This behaviour is reminiscent of the pupal eclosion rhythm in *Drosophila pseudoobscura* in DD free-run after a last photophase of more than 12 hours (Pittendrigh, 1966) (Chapter 3, D). For photophases greater than 12 hours duration the diapause maxima were about 24 hours apart, and for those photophases where T was extended to 72 hours there was evidence of a third peak, 24 hours after the second. For photophases shorter than 12 hours,

however, the first two maxima were less than 24 hours apart, the first peak always being at $T = 28$ rather than at $T = 24$ hours. This may indicate the presence of an additional component, separate from the clearly circadian one which produces 'resonance' in the system, and which, in effect, 'depresses' the expected high incidence of diapause at $T \approx 24$ hours. Since this effect does not recur in the second peak, it might represent a heavily damped component associated with dawn. These results leave little doubt, however, that the circadian system is *somehow* involved in photoperiodic time measurement in *S. argyrostoma*. Unlike similar data for *N. vitripennis* (Fig. 11.9), however, one cannot easily describe the clock in terms of dawn and dusk oscillators. One thing, however, is clear: the circadian system behaves in a manner remarkably similar to that shown by the pupal eclosion rhythm in *D. pseudoobscura* (Pittendrigh, 1966), in that its principal time cue is taken from dusk once the photophase exceeds 12 hours. In other words, the oscillator(s) involved in time measurement are 'damped out' during photophases longer than about 12 hours and then restart at dusk. With longer photophases, therefore, the photoperiodic clock in *S. argyrostoma* measures night length as suggested in the External Coincidence model. This illustrates the central importance of a 'critical night length' but suggests that the duration of the dark phase assumes a central role in *S. argyrostoma* only when it is accompanied by a light component of minimum length. The similarities between *S. argyrostoma* and those species reviewed in Section B.4 (a) now become obvious.

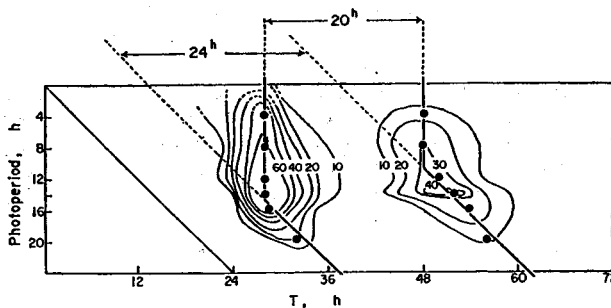


Fig. 11.11. *Sarcophaga argyrostoma*. Data from Fig. 11.10 (left) redrawn as a 'circadian topography' in which all points of equal diapause induction are joined by contours. Diapause maxima are shown as closed circles. With photophases longer than 12 hours the maxima are about 24 hours apart and appear to take their principal time cue from dusk. With photophases less than 12 hours the maxima are only about 20 hours apart and take their principal time cue from dawn (From Saunders, 1973b).

In *N. vitripennis* and *S. argyrostoma* the intervals between successive peaks of high or low diapause were about 24 hours, suggesting that the period (τ hours) of the circadian oscillator(s) involved in photoperiodic timing was very close to that value. In the spider mite *Tetranychus urticae*, however, τ appeared to be much shorter. For example, working with a Dutch strain (52°N) a large series of experiments was conducted at 19°C under regimes comprising photophases held constant at 1 to 24 hours, in cycles up to 72 hours in length. About four peaks of high diapause incidence were recorded with an inter-peak interval of about 20 hours (Fig. 11.12) (Veerman and Vaz Nunes, 1980; Vaz Nunes and Veerman, 1986a). A circadian topography revealed that a 'dusk' oscillator was involved, taking its time cue from the light off signal of each photophase. In another large series of experiments, Vaz Nunes et al. (1990b)

studied 10 latitudinal strains of the mite, from Thessaloniki in the south (40.5°N) to St Petersburg in the north (60°N). Critical night lengths for diapause induction were strongly correlated with latitude, varying from 13.25 h in Thessaloniki to 7.75 h/24 in St Petersburg. On the other hand, Nanda-Hamner inter-peak intervals, although ranging from 21.5 h in the south to only 17.75 h/24 in the north, were only weakly correlated with latitude. It was also shown, for the Russian strain, that inter-peak intervals for diapause *termination* were about 1.5 hours shorter than that for diapause induction (Veerman and Koveos, 1989). Although these experiments clearly indicated some involvement of the circadian system in photoperiodic time measurement, the form of that involvement was not clear. This will be considered further in Chapter 13.

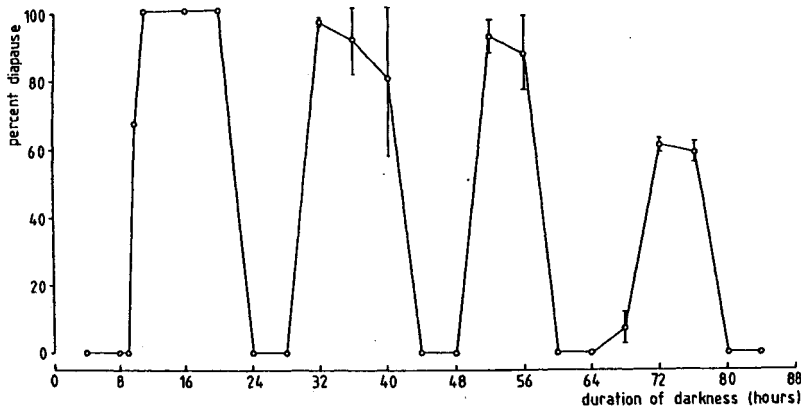


Fig. 11.12. 'Positive' Nanda-Hamner experiment with the spider mite *Tetranychus urticae* (photophase 8 hours) showing four peaks of diapause about 20 hours apart (From Veerman and Vaz Nunes, 1980).

'Negative' Nanda-Hamner responses lacking characteristic maxima and minima of photoperiodic induction have been recorded for the green vetch aphid *Megoura viciae* (Lees, 1986) and several other species, notably Lepidoptera (Tables 11.2 and 11.3). Since these responses have frequently been interpreted as the manifestation of an hourglass-like timer, they will be considered in Section C. In addition – and of particular interest – are species that sometimes show 'positive' and sometimes 'negative' responses. These include the ground beetle *Pterostichus nigrity* from different latitudes (Thiele, 1977b), the cabbage butterfly *Pieris brassicae* at different stages of diapause induction and termination (Dumortier and Brunnarius, 1981; Claret, 1985), and about 10 species showing 'positive' and 'negative' responses at different temperatures (Table 11.3). The problems raised by these observations will be further addressed in Section D.

(c) *T*-experiments

T-experiments are related to Nanda-Hamner experiments because they involve a single light phase repeated in a series of abnormal light-dark cycles. Pittendrigh and Minis (1964) introduced this experimental design as a specific test of the 'external coincidence' model, based on an understanding of the entrainment of circadian oscillations to light.

Earlier studies on the entrainment of the *Drosophila pseudoobscura* eclosion rhythm to single light pulses per cycle (Chapter 3) had shown, within certain limits, that the circadian oscillation (period, τ hours) assumed the period (T hours) of the entraining light cycle. In doing so, the discrepancy between τ and T was overcome by a discrete phase-shift ($\Delta\phi$). Furthermore, when T was less than τ the pulse fell in the late subjective night it caused a phase advance ($+\Delta\phi$), whereas when $T > \tau$ the light pulse fell in the *early* subjective night to cause a phase delay ($-\Delta\phi$). Therefore, simply by changing the period (T) of the light cycle the pulse came to illuminate different phase-points of the oscillation, the theory being that such a protocol could be used to scan the night for a particular light sensitive phase. An early application of this test to the pink bollworm moth *Pectinophora gossypiella* used single recurrent 15-minute light-pulses to define cycles ranging from $T = 20$ hours 40 minutes to $T = 25$ hours (Pittendrigh and Minis, 1964; Minis, 1965). The insect's oviposition rhythm was used as an 'independent' assay of phase within the circadian system. These values of T encompassed values both greater than and less than τ for this species (22 hours 40 minutes). The prediction was that since the light pulse would illuminate different phase points of the oscillator in different regimes it should coincide with the photoinducible phase (ϕ_i) in some regimes but not in others. However, little difference in the incidence of diapause was observed, and 15 minute pulses were considered, in hindsight, to be inadequate to effect induction.

The experiment was later repeated using light-cycles varying from $T = 20$ to $T = 27$ hours, each containing an 8-hour photophase, and with the pupal eclosion rhythm as a measure of entrainment (Pittendrigh and Minis, 1971). Once again, in the shorter cycles ($T = 20$ and $T = 21$ hours) the light-pulses, in steady state, were predicted to fall in the late subjective night, whereas in the longer cycles ($T = 24$, $T = 26$, $T = 27$ hours) they were predicted to fall in the early subjective night. Therefore, if ϕ_i occurred in the late subjective night (see Section B1 and Chapter 13) - about 5 hours ahead of the peak of eclosion - it should be illuminated when $T < 24$ hours but not when $T > 24$ hours. This would lead to diapause inhibition in the shorter cycles and diapause promotion in the longer cycles. The results of this experiment showed that although the phase of the eclosion rhythm assumed a steady state as predicted, the proportion of larvae entering diapause was 80 per cent or over in all light-cycles. Indeed, although the overall range was small (80 to 96 per cent) the sign of the dependence of diapause incidence on T was the reverse of that expected. Clearly the results were equivocal for *P. gossypiella*. A 'positive' response, as predicted from entrainment theory, however, was later found for the flesh fly *Sarcophaga argyrostoma* (Saunders, 1979a).

Cultures of larvae derived from *S. argyrostoma* maintained in long nights, and therefore 'preconditioned' for pupal diapause, were set up in a series of light-cycles from $T = 21.5$ to $T = 30.5$ hours, all containing a single 1-hour pulse of light. The phase response curve (PRC) for 1-hour pulses (see Chapter 3) was used to compute where the first pulse in the train must fall, and which T -value must be used, to illuminate each hour of the subjective night. The results (Fig. 11.13) showed that only in a cycle of $T = 21.5$ hours (LD 1:20.5), in which the light-pulse came on at Ct 20 and finished at Ct 23.5 - and therefore illuminated ϕ_i (at Ct 21.5) - were short-night, or low diapause effects obtained. In all other regimes ϕ_i fell in the dark and diapause was high (Saunders, 1979a).

Since this experiment was based on predictions arising from entrainment theory it offers strong confirmation that photoperiodic time measurement is a function of the circadian system. Also, since the postulated 'photoperiodic oscillator' was probed by a single short pulse of light *in the absence of any other photoperiodic influence*, these results offer strong circumstantial evidence for the 'reality' of the photoinducible phase (ϕ_i) late in the subjective night, close to Ct 21.5. Further tests for 'external coincidence' will be considered in Chapter 13.

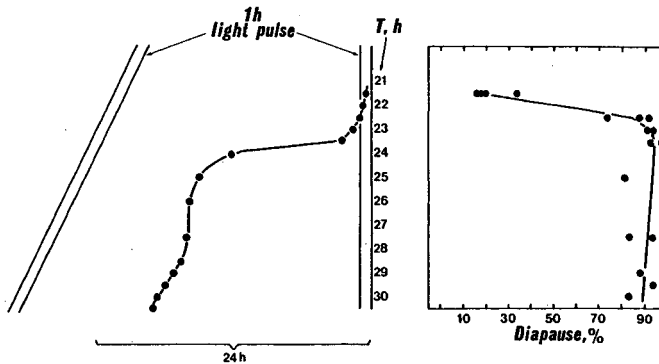


Fig. 11.13. *Sarcophaga argyrostoma*: a 'T experiment'. Left: computed phase relationships of ϕ_i (solid circles) to single pulses of light (1 hour) in cycles from $T = 21.5$ to $T = 30.5$ hours (the primary range of entrainment). Right: diapause induction in these cycles. In all T values longer than 24 h ϕ_i falls in the dark and diapause incidence is high; when T is shorter than about 22.5 h, however, ϕ_i coincides with the light pulse in each cycle and diapause incidence is greatly reduced (From Saunders, 1981a).

A 'positive' T-experiment was also recorded in a study of the photoperiodic control of antifreeze protein production in the beetle *Dendroides canadensis* (Horwath and Duman, 1984). In these experiments larvae were exposed to a series of light cycles close to 24 hours ($T = 21$ to 27 hours), all containing an 8 hour photophase. Larvae raised in cycles where T was equal or less than 24 hours were found to synthesise higher concentrations of the haemolymph protein than larvae raised at $T = 26$ or 27 hours.

Working with a 'graded' photoperiodic response controlling nymphal development in the cockroach *Parcoblatta pennsylvanica*, Wassmer and Page (1993) conducted a T-experiment with a 3 hour light phase in cycles from $T = 21$ to 27 hours. Nymphs exposed to $T = 24$ and 25 hours were found to grow slowly, whereas those in shorter ($T = 21, 22$ and 23 hours) and longer ($T = 26$ and 27 hours) cycles showed a more rapid weight gain. These results were considered to be consistent with a hypothesis that a circadian component was (somehow) involved in the photoperiodic control of growth rate.

4. Symmetrical 'skeletons' and the 'bistability' phenomenon

Powerful evidence for photoperiodic time measurement being a function of the circadian system has come from experiments using symmetrical 'skeleton' photoperiods, particularly those within the 'zone of bistability' (Chapter 3, section C).

In the entrainment of the *Drosophila pseudoobscura* pupal eclosion rhythm, a symmetrical 'skeleton' photoperiod (PP_s), consisting of two brief pulses of light n hours apart, was found to simulate many of the effects of a complete photoperiod (PP_c) of n hours duration (Pittendrigh and Minis, 1964) (Chapter 3). Similarly, symmetrical skeletons were found to mimic many photoperiodic effects, almost as effectively as the corresponding complete light pulse. These studies demonstrated the importance of the 'on' and 'off' signals of the light cycle, and in some instances a more fundamental similarity: that is, a common mechanism between photoperiodism and circadian rhythmicity.

Danilevskii et al. (1970), maintained larval cultures of the knot grass moth *Acronycta rumicis* in complete photoperiods (PP_C) of 8:16, 12:12 and 18:6 for 7 days and then released them into the corresponding skeleton photoperiods (PP_S) consisting of two 1-hour pulses of light to define the skeleton. Control groups were either maintained throughout in the complete photoperiods or released in DD. All of the larvae exposed to PP_C or PP_S 8:16 and 12:12 entered diapause as pupae, but those released into DD never produced more than 50 per cent diapause. This showed that the skeletons of the shorter photoperiods were just as effective as normal short day lengths and suggested, by analogy with the *D. pseudoobscura* data, that the clock in *A. rumicis* consisted of, or contained, an endogenous oscillator. Moreover, whilst a PP_C 18:6 produced only 8 per cent diapause, over 80 per cent of the larvae ceased development when released into the corresponding skeleton (PP_S 18:6). This suggested that the photoperiodic oscillator became re-entrained to the shorter interpretation of PP_S 6:18 (or strictly, if the duration of each pulse is also taken into account, PP_S 8:16).

Bünning and Joerrens (1960) subjected larvae of the cabbage butterfly *Pieris brassicae* to skeleton photoperiods also consisting of two 1-hour pulses of light. Pulses 6 hours apart (PP_S 6:18) and 8 hours apart (PP_S 8:16) induced a greater incidence of pupal diapause (about 42 to 45 per cent) than the DD control (about 39 per cent). On the other hand, a skeleton of PP_S 12:12 gave a lower incidence of diapause, and PP_S 15:9 only about 18 per cent. Pulses initially placed 18 and 21 hours apart, however, were once again found to be diapause promoting.

Following Pittendrigh and Minis (1964), these results may be interpreted as evidence for the operation of an oscillating system. Each skeleton photoperiod was clearly open to two 'interpretations', depending on which pulse was regarded as simulating the initiator, or the dawn signal. As in *D. pseudoobscura* and *A. rumicis* the oscillator accepted the shorter of the two intervals to signify 'day'. Thus, skeletons of PP_S 6:18 and PP_S 8:16 were equivalent to the complete photoperiods of PP_C 6:18 and PP_C 8:16, and promoted diapause. Pulses 18 and 21 hours apart, however, were 'read' as the shorter interpretation, namely PP_S 8:16 and PP_S 5:19, because the oscillator executed a ψ -jump before attaining its steady state; the results, therefore, were once again diapause-promoting. With PP_S 12:12 and PP_S 15:9, however, the skeleton light regime was clearly ambiguous, as it was with the eclosion rhythm in *D. pseudoobscura*, and diapause induction was less effective. Very similar results were obtained for the parasitic wasp *Nasonia vitripennis* and for the flesh fly *Sarcophaga argyrostoma* (Saunders, 1975b).

Another type of experiment using skeleton photoperiods was first applied to the short-day plant *Lemna purpusilla* (Hillman, 1964). Hillman maintained the plants in LL prior to their release into DD; after release they were exposed to a pair of 15-minute pulses of light defining skeleton photoperiods of either 11 or 13 hours. The results showed that the plants could distinguish between PP_S 11:13 and PP_S 13:11 depending on which interval they 'saw' first after the LL/DD transition. Whether the ensuing skeleton simulated an 11- or a 13-hour photophase, the proportion of the plants initiating flower formation was a periodic function ($\tau \sim 24$ hours) of the time at which the first pulse was seen. However, if the first interval was 11 hours the curve was almost a mirror image of that for an interval of 13 hours (Fig. 11.14). Not only did the periodic nature of the result support some form of Bünning's hypothesis, but it paralleled the remarkably similar behaviour of the eclosion rhythm in *D. pseudoobscura* for skeletons of 11 and 13 hours which Pittendrigh (1966) had called the 'bistability phenomenon' (Chapter 3).

This technique was extended to the pink bollworm moth *Pectinophora gossypiella* using the egg-hatch rhythm as an indicator of phase and 15-minute light-pulses to create the skeletons (Pittendrigh and Minis, 1971). The two alternative steady states for PP_S 11:13 and PP_S 13:11 were shown by the egg-hatch rhythm, but there was almost 100 per cent diapause in both regimes. In hindsight it was considered that 15-minute pulses, although sufficient to

entrain the overt rhythm, were probably too short to bring about the photochemical changes associated with the inhibition of diapause.

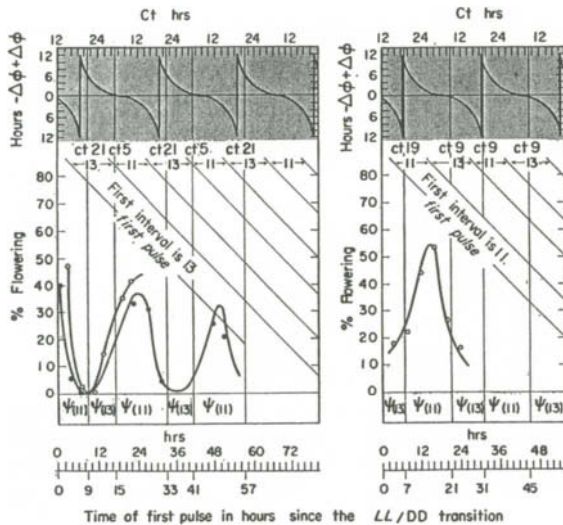


Fig. 11.14. Pittendrigh's (1966) interpretation of Hillman's (1964) data on the induction of flowering in *Lemna* by symmetrical skeleton photoperiods of PP₁₁ and PP₁₃. Flowering maxima occur when the initial conditions (the initial relationship between oscillation and the light cycle) are such that ψ_{11} is predicted. Whether ψ_{11} or ψ_{13} develops is determined by (1) the duration of the first interval, and (2) the phase of the oscillation illuminated by the first pulse. The oscillation begins at Ct 12 (the beginning of the subjective night) when the system is transferred from LL to DD (From Pittendrigh and Minis, 1971).

Hillman (1973) applied a similar technique to the green vetch aphid *Megoura viciae*. However his results seemed to suggest that this species possessed a non-circadian or hourglass-like timer, which will be considered further in Section C.

'Positive' bistability experiments with insects were first recorded with the flesh fly *Sarcophaga argyrostoma* (Saunders, 1975b, 1978a). Cultures of larvae were transferred from LL to DD and then exposed to symmetrical skeleton photoperiods (two 1-hour pulses) of either PP₁₁ (LD 1:9:1:13) or PP₁₅ (LD 1:13:1:9), with the first pulses in the train commencing at all circadian times (Fig. 11.15). These skeletons were chosen because they were 'mirror images' of each other, and if adopted by the circadian oscillator would be 'read' as short or long day, respectively. The results were similar to those of Hillman's with *Lemna*. When PP₁₁ was presented first, short-day results (high diapause) were observed when P₁ started between Ct 19 and Ct 08, and long-day results (low diapause) when P₁ started between Ct 09 and Ct 19. When PP₁₅ was presented first the results were a mirror image with high diapause between Ct 04 and Ct 18 and low diapause between Ct 18 and Ct 04. As in *Lemna* the photoperiodic response was periodic, thus providing evidence for the circadian basis of photoperiodism.

'Bistability' experiments have now been performed with 7 species of insects and one mite (Table 11.4). When 'positive', as with *S. argyrostoma*, the blow fly *Calliphora vicina* (Vaz Nunes et al., 1990) and the cabbage moth *Mamestra brassicae* (Kimura and Masaki, 1993) they provide compelling evidence that the circadian system is somehow involved in

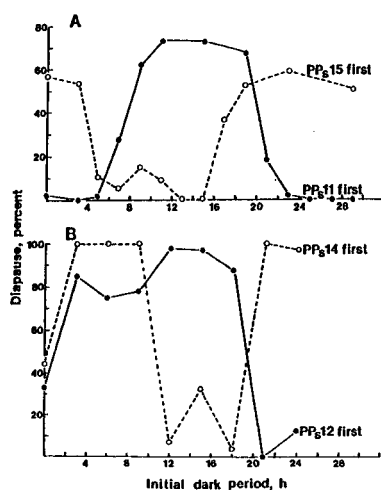


Fig. 11.15. 'Positive' bistability experiments with (A) the flesh fly *Sarcophaga argyrostoma* and (B) the blow fly *Calliphora vicina*. In A, cultures were exposed to skeleton photoperiods of LD 1:9:1:13 or LD 1:13:1:9 starting at all circadian phases (Saunders, 1975b). In *C. vicina* (B) similar data are presented for LD 1:12:1:10 or LD 1:10:1:12 (Vaz Nunes et al., 1990). See text for details.

photoperiodic time measurement. Just how the circadian system may be involved, however, will be considered in Chapter 13. 'Negative' responses to bistability experiments have been recorded in 4 insects and the spider mite *Tetranychus urticae*, in some cases at two temperatures. However, given the rather exacting requirements for this particular test to show positive, these 'negative' results do not necessarily rule out any circadian involvement.

TABLE 11.4. Bistability experiments. Table modified from Vaz Nunes and Saunders (1999).

Species	Response	Temperatures	Reference
'Positive' bistability			
<i>Sarcophaga argyrostoma</i>	Diapause induction	18°C	Saunders (1975b)
<i>Calliphora vicina</i>	Diapause induction	23.5°C	Vaz Nunes et al. (1990)
<i>Mamestra brassicae</i>	Diapause induction	20°C	Kimura & Masaki (1993)
'Negative' bistability			
<i>Pectinophora gossypiella</i>	Diapause induction	20 and 26°C	Pittendrigh & Minis (1971)
<i>Megoura viciae</i>	Ovipara production	15°C	Hillman (1973)
<i>Aphis fabae</i>	Apterisation	15 and 20°C	Vaz Nunes & Hardie (1989)
<i>Drosophila triauraria</i>	Diapause induction	15°C	Yoshida & Kimura (1993)
<i>Tetranychus urticae</i>	Diapause induction	18.5 and 22.5°C	Vaz Nunes & Veerman (1997)

C. EVIDENCE FOR HOURGLASS-LIKE TIMERS IN PHOTOPERIODISM

Tables 11.1 to 11.4 include examples of species that exhibit essentially 'negative' responses to the various protocols used to reveal circadian rhythmicity in photoperiodic time measurement. Indeed, such negative responses have been used as evidence to suggest that night length measurement is *not* a function of the circadian system, but is effected by a non-oscillatory or non-repetitive *hourglass-like* timer that measures night length from the 'dusk' signal of the daily cycle. By far the most persuasive of this evidence was presented in a long series of papers by A. D. Lees (1965, 1966a, 1968, 1973) on the green vetch aphid *Megoura viciae*, using experimental methods almost identical in design to those described in the foregoing section. These experiments, because of their importance, will be presented in some detail, along with similar data for some other species. The question whether they represent hourglass-like timers or whether there is an alternative explanation in keeping with Bünning's hypothesis will be considered in Section D.

1. Abnormal light dark cycles

The aphid *Megoura viciae* produces successive generations of wingless, parthenogenetic virginoparae during the summer months when the days are long. In the autumn when days fall below a critical point (LD 14 $\frac{1}{4}$:9 $\frac{3}{4}$) oviparae are produced which subsequently lay diapausing eggs. Experiments with abnormal light cycles demonstrated that the dark period occupies a central role in time measurement (Lees, 1965). Consequently in most of this section a long-day effect (virginopara-production) will be referred to as a short-night effect.

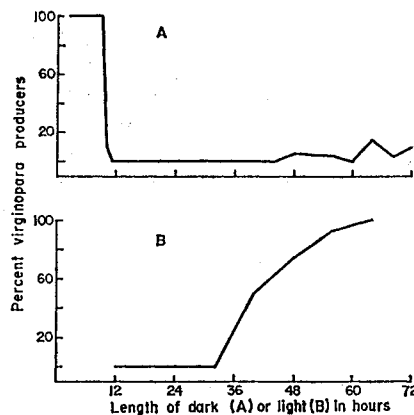


Fig. 11.16. *Megoura viciae*: the production of wingless virginoparae in photoperiodic regimes in which the length of the cycle was varied, but the duration of the photophase was held at 8 hours (A), or the scotophase at 12 hours (B). In A, virginoparae (the long day response) are produced once the dark component exceeds the critical night length (9.5 hours). The absence of periodic maxima of short day effect in longer cycles represents a 'negative' Nanda-Hamner experiment. In B, a long night is fully inductive (100 per cent oviparae) until the accompanying light period exceeds about 32 hours (Redrawn from Lees, 1965).

Figure 11.16B shows that when a night of inductive length (12 hours) was combined with a range of different light periods up to $T = 76$ hours, a low rate of virginopara-production (the long-night effect) was observed even with 'days' of up to 32 hours in duration. Only with a light period of 40 hours was the proportion of virginopara-producers raised to 50 per cent. In the converse experiment (Fig. 11.16A) a short light period (8 hours) was combined with extended nights up to $T = 60$ hours. This experiment was therefore of the resonance or Nanda-Hamner type and should be compared with those in Section B4. Application of such an experimental design to *Megoura* produced a clearly 'negative' response with no periodic maxima and minima of inductive effect. Short night effects (high virginopara-production) were thus apparent until the dark period reached $9\frac{1}{2}$ to 10 hours ($T = 18$ hours); thereafter the response was 'saturated' and all aphids became ovipara-producers. This second experiment was important for two reasons. Firstly, it demonstrated that once a critical night length ($9\frac{1}{2}$ to 10 hours) had elapsed all aphids were committed to the long-night response. Secondly, it showed that the response was the same in all cycles over $T = 18$ hours. There was no hint of a 'resonance effect' which would have produced a high level of the long-night response at $T = 24$ and $T = 48$ hours, but a low level at $T = 36$ and $T = 60$ hours. Lees, with good reason at the time, took this as strong evidence showing that time measurement in *M. viciae* was not a function of the circadian system, but comprised a non-repetitive dark period interval timer or hourglass.

Other species showing 'negative' responses to the Nanda-Hamner protocol are shown in Tables 11.2 and 11.3. Many of these are with Lepidoptera, such as the European corn borer *Ostrinia nubilalis* (Bowen and Skopik, 1976; Skopik and Bowen, 1976; Skopik and Takeda, 1986) and the Indian meal moth *Plodia interpunctella* (Takeda and Masaki, 1976). None of these 'negative' examples showed peaks of inductive effect at circadian intervals as darkness was extended, and most were interpreted in terms of an hourglass-like timer.

2. Night interruption experiments

Using a light-cycle ($T = 24$ hours) containing a dark phase just longer than the critical night length (LD 13.5:10.5), Lees (1965, 1966a) showed that 1-hour light breaks introduced systematically into the night produced two 'peaks' of short-night effect, or virginopara-production. One of these peaks occurred about 2 hours after dusk; the other was more pronounced and occurred when the light-pulse fell during the last 6 hours of the night. Pulses applied in the middle of the night were without effect. These results are very similar to those for *Pectinophora gossypiella* (Adkisson, 1964) and a number of other species (see Section B.I). However, Lees (1965, 1966) showed that the responses were unaltered if the light period accompanying the 10.5 hour night was extended to 25.5 hours (LD 25.5:10.5, $T = 36$ hours) or shortened to 8 hours (LD 8:10.5, $T = 18.5$ hours). He considered these results to be inconsistent with a circadian hypothesis.

In Lees's investigations, 'early' and 'late' light breaks seemed to have quite different modes of action. Figure 11.17a shows the effect of a one-hour light pulse applied $1\frac{1}{2}$ hours after dusk. In the first regime (LD 13.5:1.5:1:9.0) the pulse completely reversed the long-night effect and since it was followed by a short night (9.0 hours) resulted in 100 per cent of virginopara-producers. As the terminal dark period became greater than the critical night (9.5 hours), however, the effect of the early light break was overridden and long-night effects were achieved. This result demonstrated the importance of an uninterrupted long night, and showed that the effects of an early-pulse were *reversible*. In another series of experiments (Fig. 11.17b)

a night interruption was followed by a constant dark period of more than inductive length (12 hours), but the hours of dark preceding the pulse were systematically varied. It can be seen that the terminal 12 hours of darkness function as a long night (producing 100 per cent ovipara-producers) until the light pulse fell between the 7th and 10th hours after dusk, where it functioned as a *late* interruption. The effects of such a late interruption were *irreversible* because the short-night effect so produced could not be undone by the terminal 12 hours of darkness. Early and late points of light sensitivity also differed in their action spectra (Chapter 14): the early point showed a pronounced blue sensitivity with a peak at 470 nm, whereas the late night point was also sensitive in the red (Lees, 1971 a).

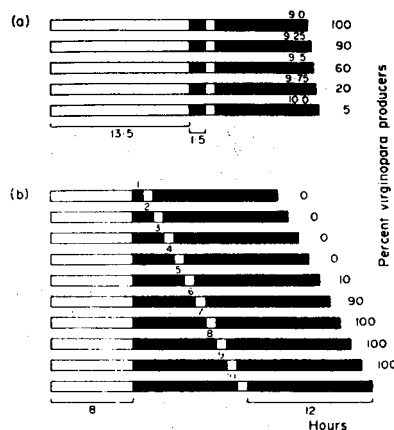


Fig. 11.17. Night interruption experiments in *Megoura viciae* showing the differences between 'early' and 'late' light breaks. In (a) a 1 hour light pulse 1.5 hours after dusk is followed by a variable dark phase. When this dark phase exceeds the critical night length (9.5 hours) short day effects (low virginopara production) ensue, demonstrating the *reversibility* of the inductive effects of a light pulse early in the night. In (b) a 1 hour light pulse scans the night but is in all cases followed by a dark phase longer than the critical night length (12 hours). When the pulse falls early in the night its long day effects are overridden by the following 12 hours of darkness; when the pulse falls later in the night, however, the long dark phase which follows it fails to reverse the long day effect (From Lees, 1970).

Lees interpreted these night interruption experiments as evidence for an hourglass timer in *M. viciae*. Very similar results for the flesh fly *Sarcophaga argyrostoma*, however, may be interpreted in terms of a circadian or oscillatory clock (see Chapter 13).

Night interruption experiments in cycles with a greatly extended night (i.e. Bünsow experiments; see also Section B2) have also failed to produce evidence for an oscillatory clock in *M. viciae* (Lees, 1966a). Figure 11.18 shows the effect of introducing a one-hour pulse of light at 4 hour intervals into the 64 hour 'night' of an LD 8:64 cycle. In all but one position, strong long-night effects (100 per cent ovipara-production) were produced. The single exception was when the pulse fell 8 hours after dusk; here it completely reversed the response. There was no evidence of further points of sensitivity at circadian intervals (i.e. at Zt 40 or Zt 64), so that the results were not obviously consistent with an oscillatory hypothesis. At first sight the results were also inconsistent with an hourglass, since all regimes contained a 'night' in excess of 9.75 hours and a high incidence of the long-night effect (ovipara production) would have been expected wherever the scanning pulse fell. The pulse falling 8 hours after

dusk, however, presumably acted as a 'late' night interruption and rendered the remaining 55 hours of darkness ineffective. The pulse falling at Zt 64 might have been expected to act as an 'initiator' of an asymmetric skeleton defining the start of a 'main' photoperiod, thereby reversing the long-night effect. Lees (1971b) showed, however, that a pulse falling in this position can only act as a 'main' photoperiod when it becomes longer than 4 hours. The function of this 'main' light component was not merely to delimit the accompanying darkness, but also to prepare the system for the timing process that began at dusk; in other words, it served to "turn the hour-glass over". In order to do this it had to be in excess of 4 hours, otherwise the accompanying 'critical night length' became longer.

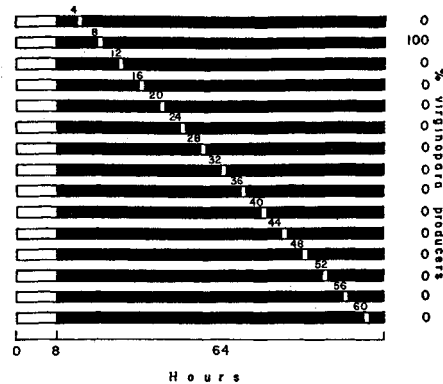


Fig. 11.18. 'Negative' Bünsow experiment with the aphid *Megoura viciae*, using a cycle of LD 8:64 ($T = 72$ hours) with the long night systematically interrupted by a 1 hour light pulse. Long day effects (high virginopara production) are only observed when the supplementary pulse falls 8 hours after dusk, a position that constitutes a 'late' interruption, which is irreversible by a subsequent long night. Later pulses fail to generate alternating maxima and minima of long and short day effect (From Lees, 1970).

The hourglass timer in *M. viciae* was thought to comprise a linked sequence of four reactions distinguished on the basis of their responses to light breaks (Lees, 1968). These will be described in Chapter 13.

3. Symmetrical 'skeleton' photoperiods

Hillman (1973) investigated the *Megoura* system using ambiguous symmetrical skeleton photoperiods in experiments of the 'bistability' type (see Section B4(b)), previously applied to the short-day plant *Lemna perpusilla* (Hillman, 1964; Pittendrigh, 1966)(Chapter 3). In these experiments, 'stock' cultures of virginoparae were raised in LL and then transferred to a number of skeleton regimes formed from two one-hour pulses of light per 24 hour cycle. The two main skeletons used were LD 1:9:1:13 and LD 1:13:1:9 (PP_s 11:13 and PP_s 15:9). In *Lemna perpusilla* and *Drosophila pseudoobscura* the steady-state phase adopted by the circadian oscillation when presented with such 'ambiguous' regimes was found to depend in part upon which interval was seen first. Therefore, in the present experiments - and if *M. viciae* possessed an oscillatory timer - those aphids transferred from LL into LD 1:9:1:13 should have 'read' the 9-hour interval of dark as 'night' and, because 9 hours is below the critical night length, produced a high proportion of virginoparae. Conversely, those aphids transferred from

LL into LD 1:13:1:9 should have responded with a low incidence of virginoparae. In additional experiments the aphids were transferred from LL into an initial 12-hour period of darkness before they experienced the first pulse, the prediction being that the phase of the oscillator would be displaced by a full half-cycle, thereby reversing the response to the following skeleton. Results showed that each of the four regimes produced 100 per cent of virginopara-producers; consequently observation did not agree with prediction. Other experiments, in which the duration of the pulses forming the skeletons was increased from 1 hour to 2, 3 or 4 hours, also failed to confirm predictions based on an oscillatory hypothesis. For this reason the results were interpreted as evidence against even a rudimentary form of circadian timing in *Megoura*. 'Negative' bistability experiments have also been produced in three other species of insect, and in the spider mite *Tetranychus urticae* (Table 11.4).

4. The Veerman-Vaz Nunes protocol

The fourth important type of experiment used to distinguish a circadian-based clock from an hourglass-like timer (after the Bünsow, Nanda-Hamner and 'Bistability' tests) was that introduced by Veerman and Vaz Nunes in 1987. This test was based on the operation of the *photoperiodic counter* (see Chapter 12) in dark phase measurement. It recognised that an hourglass-like clock executed a *single* act of time measurement before it required to be restarted by light, whereas a circadian oscillation reset itself in darkness and was therefore capable of *multiple* acts of time measurement in an extended period of darkness. An hourglass-like clock would therefore see cycles of LD 12:12 and LD 12:36 as equivalent, whereas a circadian-based clock would record the former as a single long night, and the latter as two.

This experimental design was first applied to the problem of diapause induction in the spider mite *Tetranychus urticae* (Veerman and Vaz Nunes, 1987). At 18.5°C, the photoperiodic sensitive period lasted 12 days. When the mites were kept under continuous darkness for this 12-day period the incidence of diapause was 0 per cent. With an increasing number of long nights (LD 10:14), the incidence of diapause rose until 95 per cent of the mites entered diapause after four such cycles; clearly long nights were accumulated by the counter mechanism. When mites were exposed to cycles of either LD 12:12 ($T = 24$ hours) or LD 12:36 ($T = 48$ hours), however, it was found that both 12 and 36 hour 'nights' were recorded as *single* events, with no evidence of multiple long night measurement in the longer cycles. It was therefore concluded that the clock measuring night length in *T. urticae* was an hourglass – despite the 'positive' Nanda-Hamner results recorded earlier (Veerman and Vaz Nunes, 1980; Vaz Nunes and Veerman, 1986a) (Fig. 11.12). This apparent anomaly was resolved by proposing the "Hourglass clock – Oscillator counter" model for photoperiodic induction (Vaz Nunes and Veerman, 1982). This model will be described in Chapter 13, along with some other tests of this type.

Although application of the Veerman-Vaz Nunes protocol to the spider mite suggested that the clock was hourglass-like, the converse was surprisingly true for the aphid *Megoura viciae*. Vaz Nunes and Hardie (1993) exposed short-day born *Megoura* to cycles containing 12 hours of light coupled to night lengths of 12, 36, 60 or 84 hours, and compared the incidence of virginopara producers. Once again, if the clock was of an hourglass type, each dark phase, whatever its length, would be recorded as a *single* long night. On the other hand, if a circadian-based clock were involved, *multiple* acts of time measurement would occur in the longer dark phases. Fig. 11.19 plots virginopara production as a function of the number of long-night exposures, either during a series of LD 12:12 cycles or in those with an extended night, in each case as predicted for single or multiple long night measurements. The results clearly

demonstrated that long-night measurement was accomplished in a *repetitive* manner suggesting an oscillatory or circadian clock. Therefore - despite the weight of evidence produced by Lees in favour of an hourglass timer - the photoperiodic clock in *M. viciae* is based on, or includes, a circadian oscillator after all!

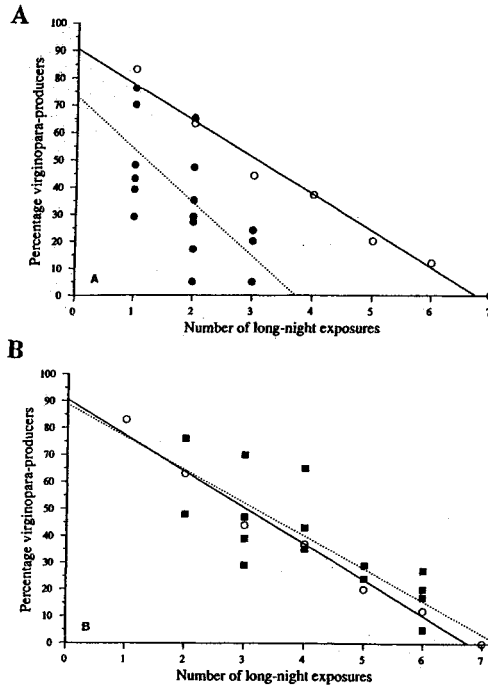


Fig. 11.19. Application of the Veerman-Vaz Nunes principle to experiments on the aphid *Megoura viciae*, showing the percentages of virginopara producers plotted against the number of long nights experienced during a series of LD 12:12 photoperiods (open circles) or in extended nights (LD 12:x)(solid circles or squares). In A experimental data for exposure to extended nights (i.e. LD 12:36, LD 12: 60 and LD 12:84) are plotted against the number of long-night exposures as predicted by an hourglass clock (i.e. each extended night is read as a single long night). In B they are plotted as predicted by a circadian oscillator clock (i.e. each extended night is seen as a sequence of long nights, measured modulo τ). The regression line for LD 12:x (variable darkness)(dotted) is much closer to that for LD 12:12 (solid) in B than in A, providing strong evidence that the *Megoura* clock is based on the circadian system, and for repetitive night length measurement. See text for details. (From Vaz Nunes and Hardie, 1993).

Considerable variation in the nature of photoperiodic time measurement seems to occur, however. Some species clearly show circadian-based clocks; others apparently show hourglass-like timers. Some species, like the spider mite *T. urticae*, are considered hourglasses but show some circadian responses, whereas others such as *M. viciae*, long considered to be an hourglass, apparently uses a circadian-based timer. Even more confusingly, some species (Table 11.2 and 11.3) show circadian responses under one condition but hourglass-like responses under another. These apparent anomalies will be addressed in the next section, and again when we consider photoperiodic clock-counter models in Chapter 13.

D. SPECIES SHOWING 'POSITIVE' RESPONSES IN ONE CONDITION, BUT 'NEGATIVE' IN ANOTHER

Tables 11.2 to 11.4 show a number of examples producing 'positive' and 'negative' responses to the various protocols. These apparently anomalous observations have been recorded, *inter alia*, between strains collected from different latitudes, at different stages of development, in different types of experimental design, or even with a change of diet. Above all, however, a number of species have been shown to display 'positive' and 'negative' responses at different temperatures. These observations will be reviewed briefly here.

Using the Nanda-Hamner protocol, Thiele (1977b) demonstrated 'positive' circadian resonance for a strain of the beetle *Pterostichus nigritya* from central Europe (51°N) but not from Swedish Lapland (64 to 66°N). Similarly, Takeda and Skopik (1985) found that a northern population of the corn borer *Ostrinia nubilalis* showed positive resonance at 30°C (but not at 20°C), whereas a southern population showed positive at 20°C (but not at 30°C). In *O. nubilalis* (Takeda and Skopik, 1985; Beck, 1988; Skopik and Takeda, 1986), and the large cabbage white butterfly (Dumortier and Brunnarius, 1981; Claret, 1985), 'positive' resonance was demonstrated for diapause induction, but not for its termination. Dumortier and Brunnarius (1989) also reported that a change of larval diet, from cabbage leaves to a semi-defined medium, changed the response from 'positive' to 'negative'. In addition, the spider mite *Tetranychus urticae* showed 'positive' resonance in Nanda-Hamner experiments (Vaz Nunes and Veerman, 1986a), but 'negative' responses in a T-experiment (Vaz Nunes and Veerman, 1982) and the bistability protocol (Vaz Nunes and Veerman, 1997). Apterisation in the black bean aphid *Aphis fabae* also showed 'positive' Nanda-Hamner results, at least at 20°C (Hardie, 1987b), but 'negative' responses for bistability (Vaz Nunes and Hardie, 1989).

An apparent change from circadian to hourglass-like responses with a change in temperature was first reported for the flesh fly *Sarcophaga argyrostoma* (Saunders, 1973b). When larvae were exposed, at 20 or 22°C, to Nanda-Hamner cycles (12 hours of light coupled to dark phases of between 4 to 60 hours), peaks of high pupal diapause were observed at T = 24-28, T = 48 and T = 72 hours, and 'troughs' of low diapause at T = 36 and 60 hours. At 16°C, however, the incidence of pupal diapause rose as darkness passed the critical night length, but then remained uniformly high in all longer values of T. This result was reminiscent of an hourglass-like response in, for example, the aphid *Megoura viciae* (Fig. 11.16). Superficially it suggested that 'positive' resonance indicating circadian involvement gave way to a 'negative' hourglass-like response merely with a fall in temperature. Similar experiments were later conducted in a range of temperatures between 14 and 28°C, with increments of 2°C (Saunders, 1982). At the lower temperatures tested, the 'troughs' of low pupal diapause close to T = 36 and 60 hours 'filled in' to approach 100 per cent diapause. Conversely, at the higher temperatures, the proportion of insects entering pupal diapause fell to very low values (less than 20 per cent), but the peaks at T = 24, 48 and 72 hours remained (Fig. 11.20).

Similar observations have now been made in about 9 insects and a mite (Table 11.3). In nearly all of these examples 'positive' resonance suggesting circadian involvement gave way to 'negative' or hourglass-like responses at the lower temperatures tested. The interpretation of this result was that temperature affected the *expression* of the circadian oscillators somehow involved in photoperiodic time measurement.

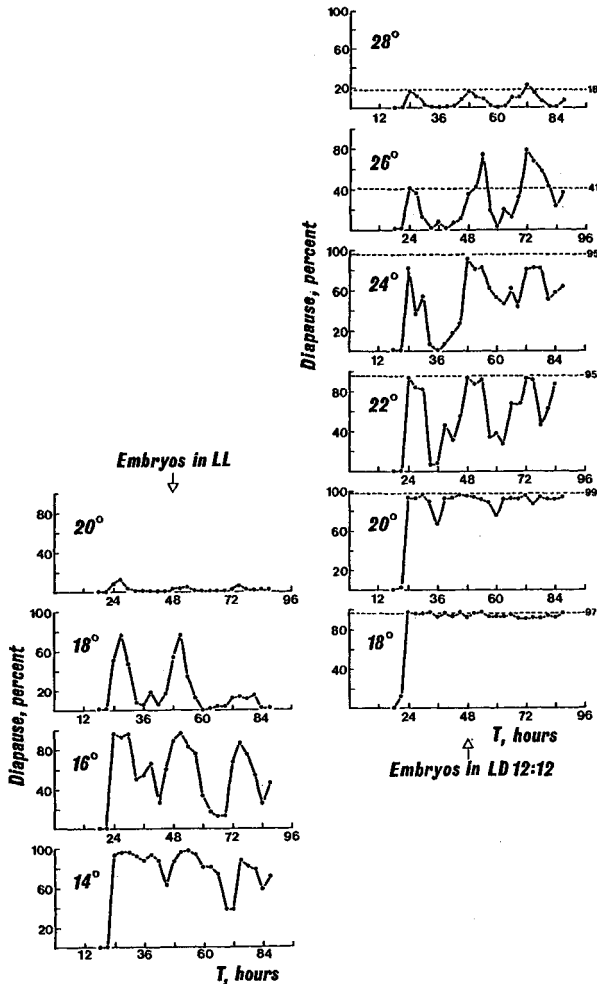


Fig. 11.20. Effects of temperature on pupal diapause induction in cultures of the flesh fly *Sarcophaga argyrostoma* in Nanda-Hamner experiments (photophase held constant at 12 hours; duration of darkness varied to give T values up to 84 hours or more). Left hand panels: larval cultures produced by female flies exposed to constant light; Right hand panels, ditto but exposed to LD 12:12 during intrauterine development. In both series, 3 peaks of high pupal diapause are evident. These are of low 'amplitude' at higher temperatures, but climb to 100 per cent diapause at lower temperatures, with the 'troughs' of low diapause gradually filling in to give an hourglass-like response. Horizontal dotted lines show the incidence of diapause in continuous darkness (From Saunders 1982).

E. HOURGLASS-LIKE CLOCKS AS HEAVILY DAMPED CIRCADIAN OSCILLATORS

It is entirely possible that insect photoperiodic clocks contain both circadian and hourglass components and that their (separate) expressions depend on such factors as

temperature, stage of development, diet or geographical origin. Indeed, models for complex multi-component clocks will be examined in Chapter 13. However, a switch from one type of clock (circadian) to another (hourglass) with changes in the environment seems to be an unnecessarily complicated interpretation of the data. Notwithstanding the obvious complexity of photoperiodic time measurement (see Chapters 13 and 16), it is surely advantageous to suggest that the circadian oscillators undoubtedly involved in such time measurement express their circadian properties ('positive' responses) in some conditions, but in others – for example, low temperature – seem to operate as hourglasses. Such changes could occur, for example, if hourglass-like timers were merely heavily damped circadian oscillators, as first suggested by Bünning (1969). With this view, low temperature, high latitude, change in diet, or diapause termination as opposed to its induction, might all affect the rate of damping of the oscillators involved. This more parsimonious interpretation of the data reflects the very many close similarities between clearly circadian (self-sustained) oscillators and apparent hourglasses: they are essentially the same, only differing in their damping coefficient.

The idea that the circadian component(s) of the insect photoperiodic clock may show varying degrees of damping - with hourglass-like clocks being heavily damped oscillators - was tested by computer simulation in a series of papers (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b).

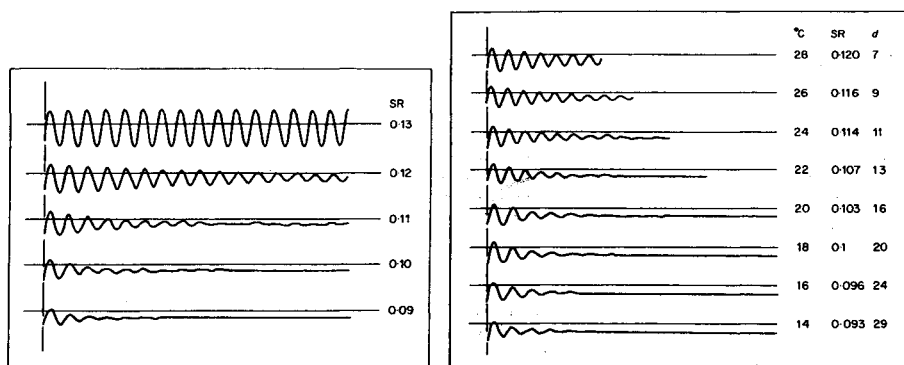


Fig. 11.21. A damped oscillator model for the photoperiodic clock (see text for details). Left – the effect of synthesis rate (SR) on the oscillation. When SR is high (0.13) the oscillation is self-sustained; when it is low the oscillation rapidly damps out. The addition of a threshold (horizontal line) shows that at lowest values of SR the oscillation damps below threshold after one cycle simulating an 'hourglass-like' time course. Right – the effect of simulated temperature (14 to 28°C) on a damping oscillation showing 'hourglass-like' responses at low temperature but increased sustainability at higher temperature (From Lewis and Saunders, 1987).

Using a simple control systems feedback model, originally developed for circadian rhythmicity (Gander and Lewis, 1979; Christensen et al., 1984; see also Chapter 7), parameter values were chosen to give a more or less damping oscillator at a simulated 'normal' temperature of 18°C. Damping was achieved by adjusting the synthesis rate (SR) of the hypothetical oscillating chemical (c). When SR was high (e.g. 0.13), and all other variables held constant, the resulting oscillator was fully self-sustaining. When SR was lower than 0.13 it resulted in a gradual damping. When SR reached 0.09 the oscillator damped out rapidly within a few cycles. With the addition of an arbitrary threshold, the oscillator with the lowest

SR value (0.09) could be made to behave as an 'hourglass' (Fig. 11.21A). Since temperature had a positive effect on SR, high simulated temperatures (e.g. 26 and 28°C) gave rise to more sustained oscillators than those at low temperatures (18 to 14°C) (Fig. 11.21B). Lewis and Saunders (1987) give details of the other parameters involved in this model.

The damping oscillator was used to simulate photoperiodic induction according to the principles of 'external coincidence' (see Section B1 and Chapter 13). The model assumed that a light-sensitive phase (ϕ_i) occurred when the oscillating chemical (c) fell through the threshold, late in the subjective night. If ϕ_i fell in the dark of each cycle, as in short days, a product (INDSUM) accumulated through the sensitive period to give a final 'incidence of diapause'. If ϕ_i was illuminated, as in long days, INDSUM was not synthesised and diapause fell to zero. Suitable selection of variables in the model led to simulations of photoperiodic responses showing all the characteristics of naturally occurring, temperature dependent, response curves (Fig. 11.22). This simple model, later elaborated (Chapter 13), was therefore capable of simulating all types of PPRC (Saunders and Lewis, 1987a), with heavily damped oscillators accounting for apparent hourglass-like responses. The model was also used to simulate both 'positive' and 'negative' responses in Bünsow and Nanda-Hamner experiments (Saunders and Lewis, 1987b). Essentially the same idea was proposed independently by Vaz Nunes and Hardie (1987) who suggested that an apparent hourglass timer in the black bean aphid *Aphis fabae* could be an 'instantly damping' circadian oscillator. These arguments are not merely 'pencil and paper' models: they are greatly strengthened by extensive computer simulations. They add weight to the idea presented here that *all* photoperiodic clocks are based on, or contain, circadian oscillators. Hourglass-like responses are merely manifestations of heavily damped circadian oscillators and not some completely different kind of timer.

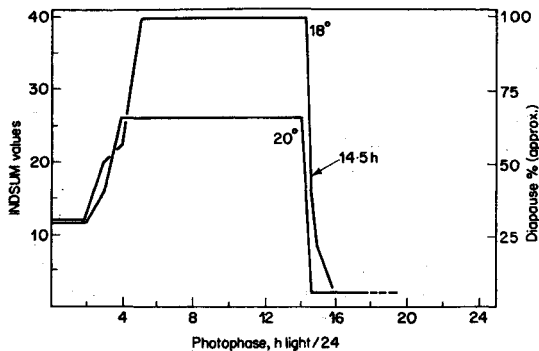


Fig. 11.22. Computed photoperiodic response curves at two temperatures (18 and 20°C) using the damped circadian oscillator model for the photoperiodic clock (see text for details). Both curves show a high diapause incidence (INDSUM value) at strong short day length (5 to 14 hours per day) and a drop in diapause at very short day lengths and in darkness. The incidence of diapause is higher at the lower temperature, but the value of the critical day length (14.5 hours per day) is little affected. Simulated responses resemble 'real' data (From Lewis and Saunders, 1987).

Justification for the proposal that damping oscillators are involved in insect photoperiodic time measurement comes from an increasing number of examples of apparent damping in overt behavioural rhythms, such as pupal eclosion (Chapter 3, I 3), especially

among strains isolated from more northerly latitudes. Direct evidence for heavily damped components in photoperiodic timing must await characterisation of the covert systems involved, but circumstantial evidence for their involvement was suggested by application of the Veerman-Vaz Nunes protocol to diapause induction in the flies *Sarcophaga argyrostoma* and *Calliphora vicina* (Saunders and Lewis, 1988).

For *C. vicina*, more compelling evidence for repetitive long night measurement, and for slowly damping oscillators, was obtained by Vaz Nunes et al. (1990). In these experiments, adult flies were maintained under continuous light (LL) for 0 to 10 days after eclosion, before being transferred to continuous darkness (DD) for the remaining days until eggs were deposited on days 10 or 11. The eggs were allowed to hatch and the resulting larvae maintained, in darkness, at 11 to 12°C to determine the proportion entering diapause. Results showed that about 5 per cent of the larvae entered diapause when adult flies had experienced three days in DD, but this rose to 100 per cent after 6 days in darkness. About 4 to 6 'cycles' in darkness were therefore sufficient for maximum induction of diapause, suggesting that 'days in continuous darkness' were accumulated by the photoperiodic mechanism. This strongly suggested that the photoperiodic clock in *C. vicina* comprised or contained circadian oscillator(s) that persist for at least 5 cycles in darkness before damping.

Models for photoperiodic time measurement incorporating such components will be discussed further in Chapter 13, after consideration of the photoperiodic counter.

ANNOTATED SUMMARY

1. This chapter addresses the evidence for (and against) the involvement of circadian rhythmicity in photoperiodic time measurement, within the framework of Bünning's general hypothesis.
2. Asymmetrical 'skeleton' photoperiods, or night interruption experiments in which the dark phase of an otherwise inductive cycle is systematically perturbed by a short light pulse, frequently show two phases of diapause reversal, one in the early subjective night and one in the late subjective night. Such experiments led to the development of the 'external coincidence' model for time measurement (see also Chapter 13) which is based on circadian entrainment theory.
3. Night interruption experiments in extended nights (e.g. LD 12:60), or Bünslow experiments, may give rise to points of diapause reversal (long night effect) at circadian intervals in the long dark period. Such results strongly indicate the involvement of the circadian system. 'Negative' results have also been recorded.
4. A phase response curve (PRC) for the postulated photoperiodic oscillator has been measured in at least one species (*Sarcophaga argyrostoma*) using three-point 'skeletons'. It shows a Type 1 PRC with phase delays ($-\Delta\phi$) in the early subjective night and advances ($+\Delta\phi$) in the late subjective night.
5. Independent variation of 'day' and 'night' in abnormal light-dark cycles has, with a few exceptions, shown the central importance of night length measurement in photoperiodic timing.
6. Nanda-Hamner or 'resonance' experiments, in which the light phase is held constant and darkness varied widely to give cycles up to 72 hours or more, have been widely applied to insects. 'Positive' results show alternate peaks and troughs of short day response (e.g. diapause induction) at circadian intervals as darkness is lengthened through two, three or more cycles of τ . Some 'negative' results, often interpreted as evidence for some sort of hourglass-like timer have also been recorded.

7. 'T-experiments' in which a short light pulse is coupled to a variable length of darkness in cycles close to the primary range of entrainment (i.e. $T = 21$ to 28 hours) sometimes produce results which may be interpreted in terms of entrainment, and specifically in terms of 'external coincidence'.
8. Powerful evidence for circadian involvement in photoperiodic time measurement may be shown in 'bistability' experiments in which insects are subjected to two short pulses per cycle (symmetrical skeletons) where the interval between them is close to $\tau/2$ (see also Chapter 3).
9. The evidence for some sort of non-oscillatory or hourglass-like timer, frequently arising from 'negative' Bünsow, Nanda-Hamner, bistability or T-experiments, is reviewed. Some species show both 'positive' and 'negative' responses at different temperatures, at different latitudes, at different stages of development, or on different diets.
10. It is proposed that apparent hourglass-like timers are merely heavily damped circadian oscillators, with the damping coefficient affected by environmental and developmental factors. Computer simulations have been used to explore this possibility. This parsimonious hypothesis states that circadian based photoperiodic clocks and apparent hourglass timers are essentially the same. How circadian oscillators may be involved in photoperiodic time measurement is considered in Chapter 13.

This Page Intentionally Left Blank

CHAPTER 12

THE PHOTOPERIODIC COUNTER

When I do count the clock that tells the time. William Shakespeare

CONTENTS

Introduction	377
<i>A. The Cumulative Effects of Photoperiod</i>	378
<i>B. Interactions between the Photoperiodic Counter and Environmental Variables</i>	383
1. Temperature	383
2. Latitude	388
3. Feeding	389
<i>C. The 'Programming' of the Central Nervous System by Photoperiod</i>	392
1. Formal models for photoperiodic summation	392
Annotated Summary	393

INTRODUCTION

THE photoperiodic response of an insect enables it to distinguish autumnal short days from the long days of summer (or long nights from short nights) to produce seasonally appropriate switches in metabolism such as the induction of an over-wintering diapause stage or, in aphids, an egg laying morph. At a formal level, this complex procedure comprises at least two processes. The first is the photoperiodic *clock* (see Chapter 11) which 'measures' night length; the second is a system, here called the photoperiodic *counter*, that accumulates successive inductive cycles during the insect's sensitive period (Chapter 10) to some sort of internal threshold which triggers the endocrine effectors (Chapter 9). Although it is possible to separate the workings of the counter from the clock (see below), it is probable that the two components are interdependent aspects of the same inductive mechanism. The clock and counter probably reflect aspects of the insect's circadian system that are entrained to the sequence of light/dark cycles experienced during the sensitive period. They are equivalent to the 'oscillator link' and 'memory link' of Goryshin and Tyshchenko (1974) that lead to the 'neuro-endocrine link' or effector system.

This chapter will examine the formal properties of the photoperiodic counter in detail, particularly its interactions with temperature, latitude and diet.

A. THE CUMULATIVE EFFECTS OF PHOTOPERIOD

In some species a single inductive cycle is sufficient to produce a measurable effect on the photoperiodic response. In the phantom midge *Chaoborus americanus*, for example, a single long day (LD 17:7) is sufficient to reactivate about 30 to 35 per cent of the diapausing larvae (Bradshaw, 1969a). In most, if not all, species, however, a *number* of such cycles are required. It is clear that the programming of the central nervous system for subsequent development or diapause involves not only the measurement of night length or day length by the photoperiodic clock, but the summation of successive cycles to a point at which induction can occur. The summation of light-cycles is therefore an integral part of the photoperiodic response, and the observation that the number of cycles required may be temperature-compensated (Saunders, 1966a, 1971; Goryshin and Tyshchenko, 1970) has led to the concept of a 'photoperiodic counter'. The interaction between this 'counter' and the rate of development during the sensitive stage - and hence with environmental factors such as temperature and nutrition - has facilitated the formal analysis of many features of the photoperiodic response.

The transfer of insects from long to short day cycles during development and *vice versa* may be used to map out the sensitive period and to determine the number of inductive cycles required for diapause induction. It may also provide information about the relative importance of long day and short day cycles in the inductive process. It should be noted, however, that the reversal of photoperiod from long to short, or *vice versa*, may produce different results from the exposure to a different number of light cycles against a non-periodic background such as continuous darkness. Examples of both types of experiment are to be found in the literature.

Early data for the oriental fruit moth *Grapholitha molesta* (Dickson, 1949) clearly indicated the cumulative effects of photoperiod. In experiments in which only part of the larval population entered diapause those larvae that emerged from the fruit first were found to be less likely to enter diapause than those larvae that emerged later. An interpretation of this observation might be that a longer larval sensitive period resulted in a greater *number* of inductive cycles being experienced and consequently a higher incidence of diapause. Working with the induction of pupal diapause in the silk moth *Antheraea pernyi*, Tanaka (1950a) transferred larvae from long day length (LD 18:6) to short day length (LD 9:15), and *vice versa*, at different stages of development. The results indicated that the sensitive stage extended at least back to the second instar, and that diapause inhibition by long days was 'stronger' than diapause promotion by short days. A cumulative effect of both long and short day cycles was also apparent. Williams and Adkisson (1964) demonstrated a similar cumulative effect of long days for the termination of diapause in this species. Previously chilled diapausing pupae maintained for 16 weeks at LD 12:12 did not break diapause, whereas those maintained at LD 16:8 for 1 week showed almost 50 per cent reactivation and those maintained at LD 16:8 for 4 weeks showed about 90 per cent reactivation. Similar results for the induction and termination of diapause in the European corn borer, *Ostrinia nubilalis*, were reported by Beck et al. (1962) and Beck and Alexander (1964); the rate of 'diapause development' in long days was found to be five times as rapid as in short days. The rate of reactivation of long day larvae was also the same when kept throughout at LD 16:8 as when maintained at LD 16:8 for the first 10 days and then transferred to DD. This result, like that described for *Nasonia vitripennis* is, incidentally, cogent evidence for an endogenous oscillator controlling the inductive process (see Chapter 13).

Evidence for the summation of both long and short-day cycles was also obtained for the knot grass moth *Acronycta rumicis* (Tyshchenko et al., 1972). For a population of this moth

from Belgorod (50°N) these authors showed that the 'critical number' of short or long day cycles (i.e. the number of cycles required to produce 50 per cent diapause) was about 10 to 11 (Fig. 12.1). For a population from Sukhumi on the Black Sea coast (43°N), however, the critical number of long days was 6 to 7, whereas the critical number of *short* days was 16 to 18. Once again, long days were clearly more 'effective' than short days.

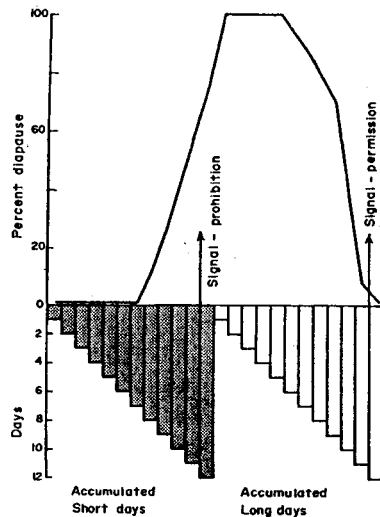


Fig. 12.1. The summation of photoperiodic cycles in the knot grass moth *Acronycta rumicis* (Belgorod strain), showing the photoperiodic counter. The graph shows changes in the number of diapausing pupae against the accumulation of short days (dark columns) or long days (light columns). The abscissa shows the duration of larval development in days (24 days at 21°C). After summing the effects of 11 short day cycles the photoperiodic counter gives a signal for diapause; after summing 11 long days the counter gives a signal for development without diapause. Only the signal that is seen last is effective (From Tyshchenko et al., 1972).

Although both short days and long days are accumulated in *A. rumicis*, in other species this may not be so. In the cabbage moth *Mamestra* (= *Barathra*) *brassicae*, for example, only short day 'information' was stored, even if short and long day photoperiods were interspersed during the sensitive period (Goryshin and Tyshchenko, 1973). On the other hand, in the aphid *Megoura viciae*, the promotive effects of long days (short nights) were clearly proportional to the number given, regardless of whether they were placed consecutively or separated by a varying number of short days (long nights) (Lees, 1971b). Short days were therefore inductive, and accumulated, in *M. brassicae*, but long days in *M. viciae*.

The parasitic wasp *Nasonia vitripennis* is particularly useful for the analysis of cumulative effects because the adult female constitutes the sensitive stage, the effects of photoperiod are transmitted through the eggs to the larvae, and eggs are deposited on practically every day of adult life. The development of the progeny - either diapause or nondiapause - is fully determined by the time the eggs are deposited within the host puparium. The daily batches of offspring, therefore, may be used to monitor the physiological state of the parent female, particularly with regard to the programming of the wasps by the photoperiodic regime.

The effect of photoperiod on the production of diapause larvae by females of *N. vitripennis* maintained in a variety of photoperiods at 18°C is shown in Fig. 12.2. Females exposed to 'strong' short day lengths (LD 6:18, LD 8:16, LD 10:14, LD 12:12 and LD 14:10) produced developing progeny for the first few days of adult life and then switched one by one to the production of diapause larvae. In a group of females the switch started on about the fifth day and was generally completed by the eleventh; the females then continued to produce diapausing offspring until they died (Saunders, 1966a). There were no significant differences between the various short-day groups with respect to the number of short-day cycles required to effect the switch. At day lengths of LD 15½:8½, LD 16:8, LD 18:6 and LD 20:4 the wasps produced very few diapause larvae, the 'switch', if occurring, being delayed until the end of imaginal life. These photoperiods, therefore, were strong long day lengths. Nevertheless, the fact that the wasps at long day length did produce diapausing offspring after a sufficient number of such cycles (>20) is of considerable interest. At photoperiods close to the critical day-length (LD 14½:9½, LD 14¼:9¼, LD 15:9 and LD 15¼:8¾) a rapid change occurred in the mean age of the females at the switch and therefore in the overall proportion of the offspring produced as diapause larvae. Figure 12.3a shows the 'required day number' (RDN) plotted as a function of photoperiod. The *required day number* is defined as the number of calendar days or photoperiodic cycles (where T = 24 hours) required to raise the proportion of diapause larvae in that day's batch to 50 per cent; it is equivalent, therefore, to the 'critical day number' of Tyshchenko et al. (1972). The RDN for short photoperiods varied between about 7 and 9 and, consequently, the overall proportion of diapause larvae was high. At long day length the RDN was over 20 days and the overall proportion of diapause larvae was low. At the critical day length the RDN showed an abrupt transition.

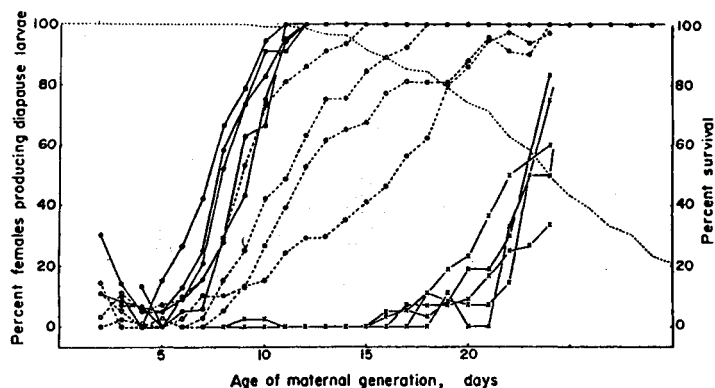


Fig. 12.2. The summation of photoperiodic cycles in the parasitic wasp *Nasonia vitripennis* at 18°C. Closed circles – under 'strong' short day lengths (6 to 14 hours/24); open circles – under 'intermediate' day lengths (from left to right: 14½ to 15¼ hours/24); crosses – under 'strong' long day lengths (15½ to 20 hours/24). The dashed line shows the survival rate of the 400 females used in the experiment (From Saunders, 1966a).

The flesh-fly *Sarcophaga argyrostoma* is also convenient for the analysis of cumulative effects and study of this species has produced data comparable to those for *N. vitripennis* described above. In *Sarcophaga*, since the sensitive period comes to an end at puparium formation, and a batch of larvae form puparia over a period of several days, it follows that those larvae which form puparia first experience fewer light-cycles than those which form puparia later (Saunders, 1971). Figure 12.4 shows that the incidence of pupal diapause rose

with an increasing number of light-cycles experienced by the larvae. Under short-day cycles (LD 8:16, LD 10:14, LD 12:12, LD 13:11 and LD 14:10) at 20°C those larvae forming puparia after 12 to 13 cycles became nondiapausing pupae, whereas those experiencing 17 to 19 cycles all became dormant; the required day number (RDN) in these particular conditions was about 14 to 15. As the photoperiod passed the critical value the RDN became higher until at long photoperiods (>14 hr/24) it could no longer be measured because all the larvae had formed puparia before the critical number of long-day cycles had been experienced. As in *N. vitripennis*, however, it is possible that long-day cycles are also inductive (in the diapause-induction sense) if a sufficient number of them are seen before the end of the sensitive period.

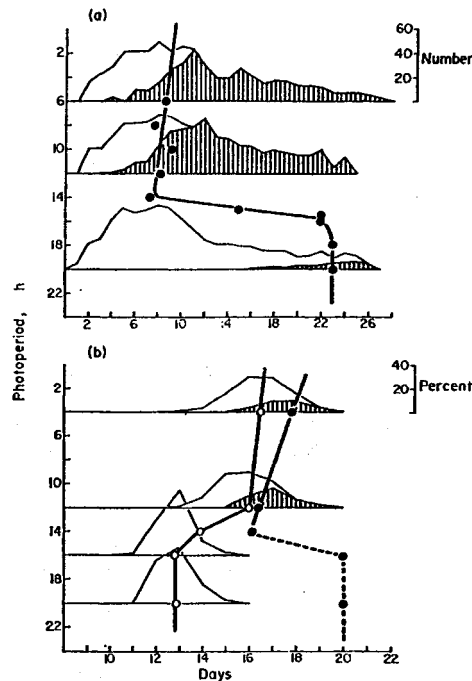


Fig. 12.3. The interaction between the required day number (RDN) and the length of the sensitive period (SP) in (a) *Nasonia vitripennis* and (b) *Sarcophaga argyrostoma*. In (a) the whole of adult life constitutes the (maternal) SP and is represented by the oviposition curve; it is essentially the same at all day lengths. The number of photoperiodic cycles required to raise the proportion of diapause to 50 per cent (the RDN), however, is low under short day lengths, but becomes abruptly greater as the critical day length is passed. Consequently, under short day length (<15 hours/24) the proportion of diapausing offspring is high, whereas under long day length (>15 hours/24) it is low. (Data from Saunders, 1966a). In (b) the period of larval development constitutes the SP. Under short day lengths larval development is protracted but under long day lengths it is significantly shorter. The RDN at short day length, however, is smaller so that a high proportion of the larvae enter diapause in the pupal instar. Under long day lengths RDN is presumed to be high so that none of the pupae become dormant. Open circles – length of sensitive period; closed circles – required day number (RDN). The polygons show the proportion of the eggs produced (a) or puparia formed (b) per day; the shaded portions represent those insects that enter diapause. (Data from Saunders, 1971, 1972).

Subsequent experiments (Saunders, 1972) showed that larvae of *S. argyrostoma* raised under long day length (>14 hr/24) developed more rapidly than those raised at short day length (Chapter 6, C.2). Consequently, the sensitive period was shorter in the long-day larvae. Figure 12.3b shows the 'antagonistic' interaction between the sensitive period and the required day number. At short day length (LD 4:20 and LD 12:12) the duration of the sensitive period (SP) was relatively long (>16 days) and the RDN short. The proportion of pupae entering diapause was therefore high because a high proportion of them were able to experience a sufficient number of photoperiodic cycles before puparium formation cut short the sensitive period. Conversely, at long day length (LD 16:8 and LD 20:4) the SP was relatively short (~13 days) and the RDN (presumably) too great to measure; consequently diapause pupae did not appear. Further evidence for this interaction between the sensitive period and the required day number will be described in the next section.

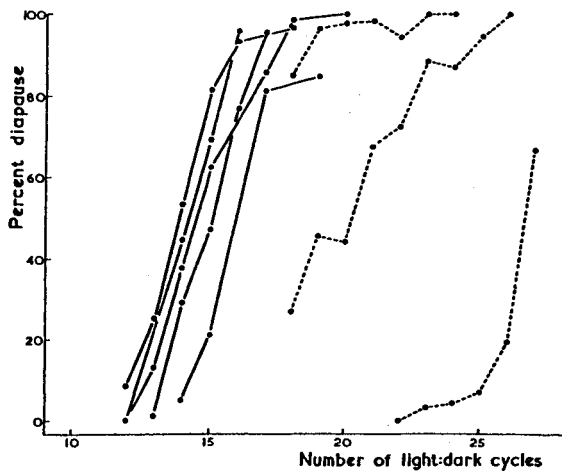


Fig. 12.4. The summation of photoperiodic cycles in *Sarcophaga argyrostoma*. Closed circles, solid line – at 20°C, from left to right, 12 to 14 hours/24; closed circles, dotted line – at 15°C, from left to right, 10, 14 and 15 hours/24. (From Saunders, 1971).

More recent observations on the accumulation of light cycles include studies on spider mites (Vaz Nunes and Veerman, 1982; Veerman and Vaz Nunes, 1984, 1987), papilionid butterflies (Shimada, 1985; Endo et al., 1986), the blow fly *Calliphora vicina* (Saunders, 1987a) and aphids (Vaz Nunes and Hardie, 1994; Hardie and Vaz Nunes, 2000). These studies generally show that an increasing number of inductive long nights experienced during the sensitive period leads to an increased incidence of diapause or the 'short-day' morph. Conversely, short nights may be accumulated to decrease the final incidence of diapause. In the tobacco hornworm moth, *Manduca sexta*, however, diapause duration is *inversely* related to the number of long nights (Denlinger and Bradfield, 1981). For example, larvae maturing in September receive a greater number of long nights than those maturing in August, yet enter a *shorter* diapause. This mechanism may serve to synchronise the population with regard to the initiation of adult development at the end of dormancy.

A photoperiodic counter may also operate for diapause termination (diapause 'development') (Numata, 1992; Koveos and Veerman, 1994). In the spider mite *Tetranychus urticae*,

for example, successive short nights were accumulated to accelerate reactivation of diapausing mites, but long nights, although slowing diapause development, did not appear to be governed by a counter (Koveos and Veerman, 1994).

B. INTERACTIONS BETWEEN THE PHOTOPERIODIC COUNTER AND ENVIRONMENTAL VARIABLES

1. Temperature

With long-day insects, the effects of low temperature and short day length tend to complement each other and result in an increase in the incidence of diapause, whereas high temperature and long day length work together to avert diapause (Chapter 10, D.1). Although direct effects of temperature on diapause induction undoubtedly exist, the above relationship between temperature and photoperiod can be interpreted, in a number of species, as an interaction between the photoperiodic counter and the rate of development. This relationship is probably widespread if not universal.

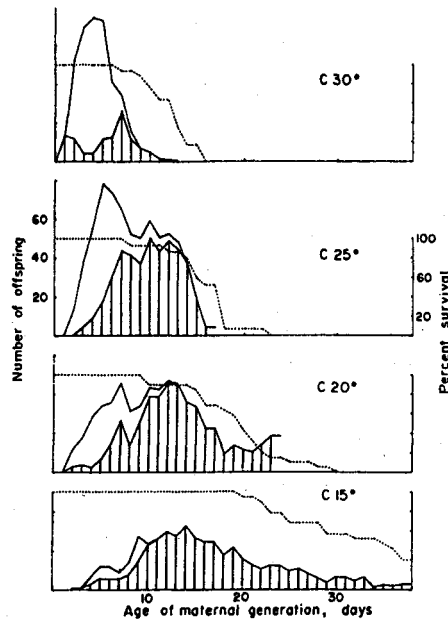


Fig. 12.5. The effect of temperature on the production of diapausing larvae by females of *Nasonia vitripennis* maintained under short day length (LD 12:12), showing its relationship to egg production. The solid line shows the rate of egg production, with the shaded portion of each polygon the proportion of the larvae subsequently entering diapause. The dotted line shows the survival rate for the females in each group. Note that temperature has a marked effect on survival and oviposition rate (equivalent to the sensitive period in this species), whereas it has a negligible effect on the number of short-day cycles needed to effect the switch to diapause (the RDN). Consequently, the proportion of offspring produced as diapause larvae falls as the temperature rises. (From Saunders, 1966a).

Females of the parasitic wasp *Nasonia vitripennis*, incubated at 15°, 20°, 25° and 30°C under LD 12:12, all reacted to the strong short day length and switched to the production of diapausing larvae within 11 or 12 days (Saunders, 1966a). The required day numbers (RDN) for the four groups were 8.4, 7.6, 8.4 and 6.9 days, respectively, and therefore showed a high degree of temperature compensation ($Q_{10} = 1.04$). Life span and the rate of oviposition, however, showed a more normal relationship to temperature. Thus, at 30°C, the wasps showed a short life span (11.5 days) and a rapid rate of oviposition with a peak 3 to 5 days after emergence. At 15°C, on the other hand, the wasps showed a protracted life (32.7 days) and a slower rate of oviposition with a peak about 14 days after emergence (Fig. 12.5). Interaction between the temperature-dependent rate of oviposition and the temperature-compensated mechanism accumulating light-cycles resulted in a high incidence of diapause at 15°C (90.9 per cent), somewhat lower at 20°C (71.2 per cent) and 25 °C (61.0 per cent), and a low incidence at 30°C (27.4 per cent).

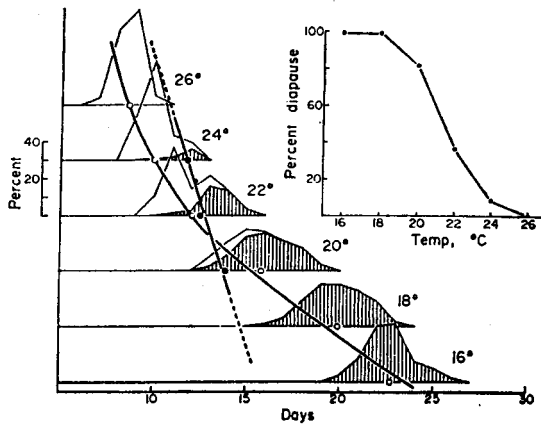


Fig. 12.6. The effect of temperature on the induction of pupal diapause in *Sarcophaga argyrostoma* under short day length (LD 10:14) showing the interaction between the sensitive period (SP; open circles) and the required day number (RDN; closed circles). The polygons show the proportion of each batch of larvae forming puparia each day; the shaded portion those larvae that became diapausing pupae. Note that the SP and RDN have different temperature coefficients. At high temperature (26 and 24°C) the SP is shorter than the RDN and few, if any, of the pupae enter diapause, whereas the opposite is true at lower temperatures (18 and 16°C). *Inset*: the effect of temperature on the proportion of diapause pupae at LD 10:14. (Data from Saunders, 1971).

A very similar relationship between developmental rate and RDN was observed with the flesh fly, *Sarcophaga argyrostoma* (Saunders, 1971). Batches of larvae were set up under short day length (LD 10:14) and at 16°, 18°, 20°, 22°, 24° and 26°C. Puparia were collected as they formed, separated from the rest of the group and incubated in the dark at 20°C. Figure 12.6 shows the pattern of puparium formation at the various temperatures with the shaded portion of the polygons representing the diapausing pupae in each batch. The length of larval development (the sensitive period) was clearly temperature-dependent, being about 9 days at 26°C and about 22 to 23 days at 16°C. At 26°C none of the pupae entered diapause whereas at 18° and 16°C practically all did so. At the intermediate temperatures (24°, 22° and 20°C) both developing and diapausing pupae were produced. The curves for puparium formation at these intermediate temperatures were in two cases clearly bimodal, with the developing

individuals in the first peak and the diapausing individuals in the second. This suggests that larvae destined for nondiapause development had a shorter larval developmental time; this might, in part, account for the increase in diapause incidence with larval age. Nevertheless, when the incidence of pupal diapause in the individual groups was plotted as a function of the number of light-cycles experienced as larvae (Fig. 12.7) it became clear that there was a 'family' of curves all with the same general and upward trend. This is compelling evidence that larvae of *S. argyrostoma* 'add up' successive light-cycles and react accordingly. At 24°, 22° and 20°C the first larvae to form puparia (after 9 to 11 cycles) showed nondiapausing pupal development, whereas those that experienced a greater number of cycles became dormant. The larvae reared at 18° and 16°C experienced 17 or more short-day cycles and all of them became diapausing pupae.

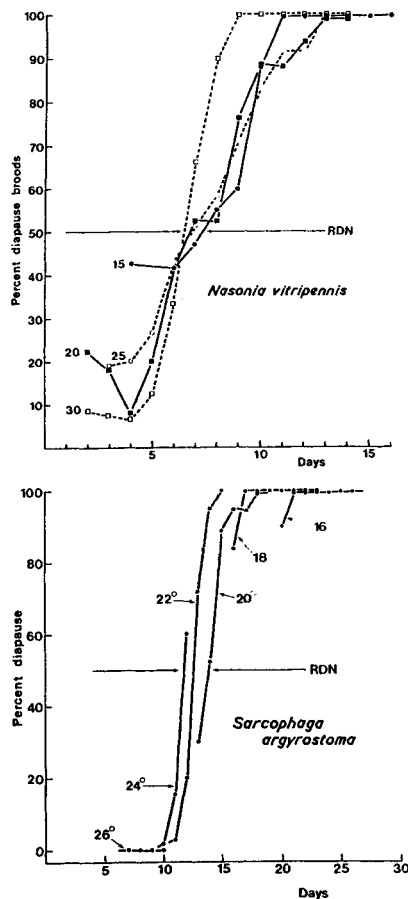


Fig. 12.7. Required day number (RDN) for *Nasonia vitripennis* and *Sarcophaga argyrostoma* under diapause inducing short days (long nights) at different temperatures. Figures plot the proportion of diapause in each day's batch (of larval progeny in *Nasonia* or pupae in *Sarcophaga*). In both species diapause incidence increases with an increasing number of long nights, and the number of long nights required to raise diapause to 50 per cent (the required day number, RDN) is practically the same at all temperatures tested. (Data from Saunders, 1966a, 1971).

Figure 12.6 shows that the rate of larval development - or the duration of the larval sensitive period - was temperature-dependent, with a Q_{10} of about 2.7; this is normal for a physiological process of this kind. On the other hand, the temperature coefficient for the number of light-cycles needed to raise the proportion of diapause pupae to 50 per cent (the RDN) is much nearer unity ($Q_{10} = 1.4$), and therefore shows a high degree of temperature-compensation. The mechanism controlling diapause induction in *S. argyrostoma*, therefore, is similar to that in *N. vitripennis*. It depends on an interaction between a temperature-dependent process (the length of the sensitive period, SP) and a temperature-compensated process (the summation of light-cycles, or the RDN). At 26°C larval development is so rapid that the larvae experience too few short-day cycles before the sensitive period is terminated by puparium formation; consequently none of the pupae enter diapause. Conversely, at 18° and 16°C, the sensitive period is so protracted that more than a sufficient number of inductive cycles are experienced before the end of sensitive period, and practically all of the insects become dormant. Figure 12.7 suggests that the required day number (RDN) at LD 10:14 is about 13 to 14, and that about 17 to 19 are required to complete the switch to diapause.

Later observations on *S. argyrostoma* (Gibbs, 1975) showed that a transfer of newly formed puparia to a higher temperature resulted in a reduction of diapause incidence, whereas a transfer to a lower temperature caused an increase. Consequently, in the experiments described above (in which all puparia were incubated at 20°C) the observed incidence of diapause at 18° and 16° was probably lower than if the insects had been kept at these temperatures throughout, and the observed incidence of diapause at 24° and 22° was probably higher. It is likely, therefore, that the temperature coefficient for the summation process is much closer to unity.

Goryshin and Tyshchenko (1970) demonstrated a similar phenomenon with the photoperiodic counter in a Belgorod strain of the knot grass moth *Acronycta rumicis*. At temperatures between 18° and 26°C the length of larval development was dependent on temperature, but the number of either long-day (LD 22:2) or short-day (LD 12:12) cycles applied at the end of larval development, and required to produce a 'critical' (i.e. 50 per cent) level of diapause, was roughly the same at all temperatures. The RDN in this species, therefore, was also temperature-compensated.

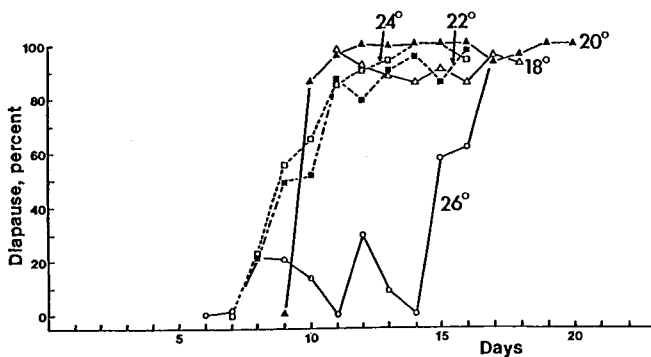


Fig. 12.8. *Calliphora vicina*. The incidence of larval diapause from egg batches deposited on successive days of adult life, and at a range of temperatures (18 to 26°C) under short day length (LD 12:12). Apart from the highest temperature, all cultures require about nine to ten short-day cycles to complete the 'switch' to the production of diapausing larvae. All larval cultures were raised in darkness at 11°C. (From Saunders, 1987a).

In the blow fly *Calliphora vicina*, larval diapause is of maternal origin (Vinogradova and Zinovjeva, 1972b; Saunders, 1987a), with the sensitive period effectively coming to an end when the eggs are laid. As with *N. vitripennis*, therefore, the incidence of larval diapause in each day's batch of progeny is a measure of the physiological state of the maternal inductive mechanism on the day of oviposition. Saunders (1987) maintained adults of *C. vicina* at temperatures of 18°, 20°, 22°, 24° and 26°C under a diapause inducing short day regime (LD 12:12). Each day's batch of eggs was collected, allowed to hatch, and the hatchlings established as larval cultures at 11-12°C in continuous darkness. It was found that temperature had a profound effect on the date of first oviposition and the rate of oviposition. The incidence of diapause in each day's batch of larvae, however, followed a 'family' of curves regardless of temperature (Fig. 12.8). Eggs laid on days 6, 7, 8 and 9 gave rise to a low incidence of diapause whereas - at all but the highest temperature (26°C) - eggs laid on day 10 and thereafter gave rise to a high incidence of diapause. Once again, as in *N. vitripennis* and *S. argyrostoma*, diapause induction was the result of an interaction between a temperature dependent process (the sensitive period) and a temperature compensated mechanism for photoperiodic summation (the RDN, or 'counter').

Although there is evidence for temperature compensation of the summation process in a number of species, there are exceptions. For example, Hodkova and Hodek (1987), working with the linden bug *Pyrrhocoris apterus*, could find no evidence for such an effect. Moreover, Kimura and Masaki (1993) found that the cabbage moth *Mamestra brassicae* required a greater number of long nights at 25° than at 20°C when the larvae were exposed to different numbers of cycles against an LL 'background'.

However, both temperature compensated *and* temperature dependent cycle accumulation has been described in three species, the aphids *Megoura viciae* (Hardie, 1990) and *Aphis fabae* (Vaz Nunes and Hardie, 1999), and the flesh fly *Sarcophaga argyrostoma* (Saunders, 1992). These observations are important because they led to the development of the 'double circadian oscillator' model for photoperiodic time measurement (Vaz Nunes, 1998) (see Chapter 13); they will, therefore, be described in some detail.

Working with the green vetch aphid *Megoura viciae*, Hardie (1990) found that the RDN for long night (LD 12:12) summation was temperature compensated - at least between 12° and 15°C - whereas that for short night (LD 16:8) accumulation was not. Similarly, for female morph and sex determination in the black bean aphid *Aphis fabae* at 12.5°, 15° and 17.5°C, the RDN for long nights was temperature compensated, but accumulation of short nights was markedly temperature dependent. In the flesh fly *Sarcophaga argyrostoma* diapause incidence in cultures exposed to different numbers of either long or short nights was studied at 16°, 18° and 20°C. In one series of experiments, larvae 'preconditioned' for a low incidence of pupal diapause by an embryonic exposure to continuous light (Denlinger, 1972) were given 1 to 17 long night cycles (LD 12:12) before transfer to DD. The resulting pupae were then assessed for their diapause or nondiapause status. In a second series, larvae 'preconditioned' for a *high* incidence of pupal diapause by an embryonic exposure to LD 12:12 (Denlinger, 1972) were given 1 to 17 *short* night cycles (LD 16:8) before transfer to DD. When results of the first series of experiments were plotted as percent diapause in each day's batch of puparia, a family of curves showing a daily increase in pupal diapause was obtained, irrespective of temperature (Fig. 12.9). In contrast, a similar treatment of the short night data revealed no such temperature compensation. Clearly in these three species, long night accumulation is governed by a temperature compensated 'counter' mechanism, whereas the accumulation of short nights is not.

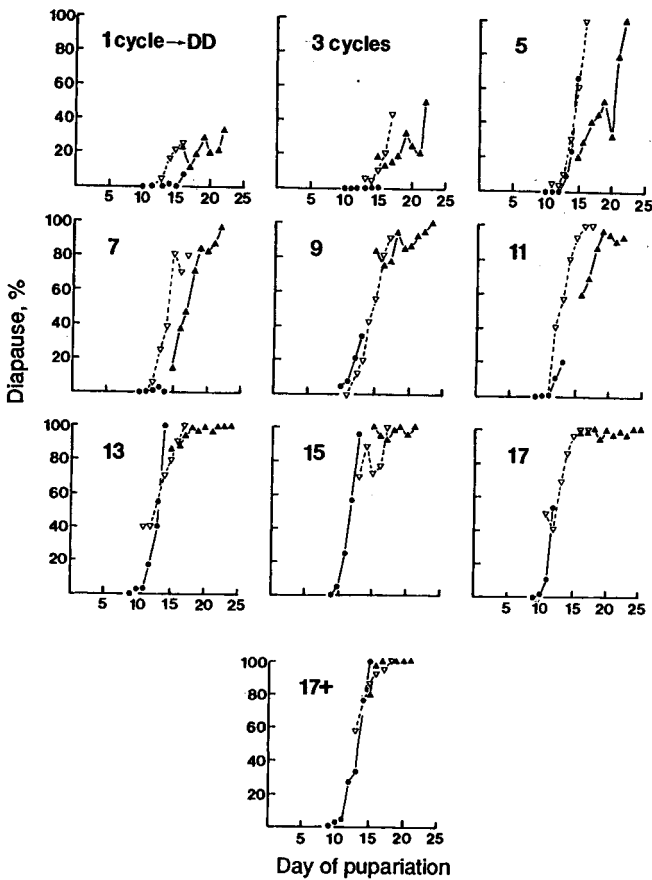


Fig. 12.9. *Sarcophaga argyrostoma*. The incidence of diapause in each day's batch of pupae in cultures of larvae transferred from 1, 3, 5 17 cycles of LD 12:12 to DD at 20°C (closed circles), 18°C (open triangles) or 16°C (closed triangles). The curves for the three temperatures nest together in families of curves demonstrating the essential temperature-compensation of the summation process (the photoperiodic counter). From Saunders, 1992.

2. Latitude

Early evidence that properties of the photoperiodic 'counter' mechanism varied according to geographical origin was provided by Tyshchenko et al. (1972) using the knot grass moth, *Acronycta rumicis* (see section A). Beach (1978) then described similar effects in three geographically distinct populations of the rock-hole mosquito *Aedes atropalpus*, originating from Ontario, Canada (45°N), Georgia, U.S.A. (34°N), and El Salvador (14°N). These strains differed in both their critical day lengths (see Chapter 10, E) and in their required day numbers. The northernmost population, for example, deposited diapause eggs after only 4 short-day cycles, but at 34°N and at 14°N the RDN had lengthened to 7 and 9 respectively. The low RDN of the Canadian strain caused it to enter diapause whenever the day length dropped into the short-day range. Because of the low value of the RDN this response was stable up to

28°C, and appropriate for an area where the transition from a favourable summer to an adverse winter was rapid and closely correlated with photoperiod. The most southerly population, on the other hand, although still capable of entering diapause, rarely did so because the prevailing high temperatures produced a sensitive period generally shorter than the 9 required cycles. At the intermediate latitude (34°N), the mechanism was more susceptible to temperature, the insects being able to lay more nondiapause eggs at high temperature when the sensitive period fell below 7 days. This strategy is particularly suitable for climatic conditions at 34°N where the onset of short days frequently coincides with a warm autumn that allows further breeding and an eventual increase in the size of the overwintering population. The observed differences in RDN are undoubtedly a product of natural selection, and have a real and clear ecological significance.

More recent observations on latitudinal effects on the counter mechanism include studies on spider mites (Veerman, 1998) and aphids (Vaz Nunes and Hardie, 1999). In the latter, black bean aphids (*Aphis fabae*) from a Scottish clone (57°N) were shown to require 2 to 4 long nights for alata production, whereas those from a more southerly clone (52°N) required 3.5 to 5 such cycles.

Latitudinal effects on diapause *termination* have also been described. For example, in the scorpionfly *Panorpa vulgaris* the number of *short* nights required for diapause development was greater at 51°N than at 48°N (Sauer, 1984). And in the spider mite *Tetranychus urticae*, diapause termination by short nights showed a lower RDN in southerly strain (Thessaloniki, Greece 40.5°N) than in one from St Petersburg, Russia (60°N).

3. Feeding

In Chapter 10 it was seen that photoperiodic responses may be modified by qualitative and quantitative aspects of nutrition. In a few insect species there is now evidence to suggest that these nutritional factors affect the balance between the required day number (RDN) and the length of the sensitive period (SP). In the parasitic wasp *Nasonia vitripennis*, for example, host deprivation caused starvation and delayed oviposition whilst the photoperiodic counter continued to function normally. The *proportion* of diapausing progeny was therefore increased. The species of host puparium offered to the wasps, however, appeared to constitute a qualitative difference that may alter the RDN. Similarly, starving the larvae of *Sarcophaga argyrostoma* may shorten the larval sensitive period with marked effects on the incidence of pupal diapause.

One of the most important factors affecting the biology of *N. vitripennis* is the availability of hosts, because host pupae provide a protein supply for the adult wasps and a place in which to deposit the eggs (Roubaud, 1917). If blow fly puparia are readily available, feeding and oviposition occur without delay. Host shortage, on the other hand, results in starvation and egg retention. In a newly emerged wasp so deprived, the few eggs that develop from larval reserves undergo a slow cycle of resorption in the ovary (Edwards, 1954; King, 1963), and only when hosts are again available can full egg production proceed.

Newly emerged females of *N. vitripennis* maintained at 18°C and under short day length (LD 12:12) were deprived of host puparia (*S. argyrostoma*) for 3, 5 and 7 days, respectively, before being supplied with two hosts per day for the rest of their lives (Saunders, 1966b). A control group was supplied with hosts daily throughout the experiment. Analysis of the progeny produced by these wasps showed that females of the control group produced about 630 offspring during a mean life-span of 32.0 days, with a peak in the oviposition rate on about the tenth day. The required day number for this group was 9.1 days, and about 73 per cent of

the progeny entered diapause. Being unable to feed, the host-deprived groups starved and were unable to develop and deposit eggs. Under the most severe conditions of starvation (7 days without hosts) eight (20 per cent) of the group died before a protein meal could be obtained. The survivors, however, showed no significant reduction in longevity or fecundity once provided access to host puparia.

Host deprivation had a marked effect on the overall pattern of diapause production by females of *N. vitripennis*. For instance, three days without a host raised the proportion of diapause larvae to 86 per cent, whereas 5 and 7 days of deprivation raised the incidence of diapause to 91 and 99 per cent, respectively. In other words, oviposition was delayed during the period of starvation but the photoperiodic counter continued to operate normally. Once again, therefore, the degree of the photoperiodic response can be attributed to an interaction between the rate of development and the RDN.

The use of puparia other than *S. argyrostoma* as host for *N. vitripennis* had quite a different effect on diapause induction (Saunders et al, 1970). With *Calliphora vicina* (= *erythrocephala*) the wasps showed a much greater survival rate and in some experiments a higher fecundity. The number of short-day cycles (LD 13½:10½ or LD 14½:9½) required to effect the switch to diapause production (the RDN), however, was also considerably increased, from about 8.0 with *S. argyrostoma* to between 12.4 and 17.7 in various experiments with *C. vicina*. A similar increase in the RDN was observed with wasps supplied with puparia of *Phormia terraenovae*. The net result was an overall *reduction* in the proportion of the progeny produced as diapausing larvae.

Wasps supplied with the smaller *C. vicina* and *P. terraenovae* were able to drill through the relatively thin puparia of these species in about 9½ and 12½ minutes respectively. Those provided with the large and thick puparia of *S. argyrostoma*, however, needed about 74 minutes to complete the drilling process. The ease in drilling and the consequent ready access to haemolymph may account for the increased longevity and fecundity shown by the wasps provided with puparia of the two calliphorines. It does not account for the increased RDN, however: wasps supplied with small puparia of *S. argyrostoma* with a puparium thickness equivalent to that in the calliphorines completed the drilling process in less than 14 minutes, showed a correspondingly high longevity and fecundity, but a relatively low RDN. The effect of *C. vicina* and *P. terraenovae*, therefore, may be a qualitative aspect of nutrition affecting the adult wasps which feed on the haemolymph exuding from the puncture in the host puparium.

As with *N. vitripennis*, denying blow flies (*C. vicina*) access to protein delayed egg production but not the accumulation of inductive light cycles (Saunders, 1987a). Flies under a diapause inducing photoperiod of LD 12:12 were provided with their first feed on meat at different times after eclosion. Regardless of whether their first protein meal was on day 2, 4 or 6 post eclosion, the incidence of larval diapause from egg batches deposited on successive days followed a similar upward curve with a RDN of about 10 in each group. However, since egg production was accelerated in those flies fed meat on day 2, and delayed in those first supplied with meat on day 6, the overall *proportion* of diapause was lower, or higher, respectively in these groups. These data indicate that the photoperiodic counter in *C. vicina* proceeds in the absence of ovarian development.

If larvae of *S. argyrostoma* are manually extracted from their larval medium before the feeding process is completed, or if the larvae are grossly overcrowded, starvation results and the small-sized larvae undergo premature puparium formation. The shortening of larval sensitive period caused a reduced incidence of pupal diapause, presumably because the number of short-day cycles they were able to experience was reduced without a correspondingly great reduction in the RDN (Saunders, 1975c). Conversely, allowing fully-grown larvae to disperse

into wet rather than dry sawdust, delayed puparium formation (Ohtaki, 1966, Ohtaki et al., 1968) and caused a corresponding increase in pupal diapause. Work by Droop (1975) and Saunders and Bradley (1984) showed that extending larval development by extracting *young* larvae from the meat for a day or two before returning them to complete their feeding, had a particularly strong diapause-enhancing effect (Fig. 12.10). This result is consistent with the idea that the early larval stages of *Sarcophaga* are more sensitive to photoperiod than the mature larvae.

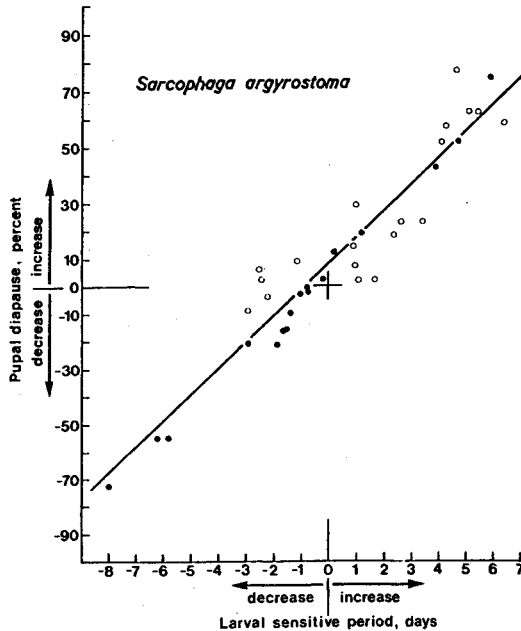


Fig. 12.10. *Sarcophaga argyrostoma*. The effect of manipulating the length of the larval sensitive period (SP) on the incidence of pupal diapause under LD 12:12. The SP was lengthened by starving young larvae for a few days, or shortened by the premature extraction of third instar larvae from their food. Open circles - 20°C; closed circles - 18°C. Note that a 1 day increase or decrease in the larval sensitive period results in a 10 per cent increase or decrease in the proportion of the larvae entering pupal diapause. (Data from Droop, 1975).

Clay and Venard (1972) described a similar effect on the induction of larval diapause in the mosquito *Aedes triseriatus*. Larvae maintained under LD 14:10 and provided with an 'adequate' diet (40 mg of pulverised Purina chow/week for 20 larvae) entered diapause; the effects of photoperiod were clearly cumulative. The incidence of larval diapause, however, was raised if the larvae were provided with an 'inadequate' diet (10 mg chow/week for 20 larvae), or kept at a lower temperature. Both of these treatments slowed development and presumably allowed the larvae to 'see' a greater number of inductive cycles before the end of the sensitive period. Working with *Aedes atropalpus*, Beach (1978) also found that prolonging the larval-sensitive period by starvation allowed the larvae to accumulate additional light-cycles, thereby inducing the resultant adults to deposit diapausing eggs even at the highest temperature tested (28 °C).

D. THE 'PROGRAMMING' OF THE CENTRAL NERVOUS SYSTEM BY PHOTOPERIOD

Photoperiodic induction is a brain-centred phenomenon (see Chapter 14) involving, in sequence, measurement of night length, accumulation of inductive cycles (by the counter), storage of this information, and its final 'translation' into hormonal signals by the endocrine effectors. Relevant photoreceptors are most frequently in the brain, the clock-counter mechanism and subsequent storage occur within the brain, and the primary endocrine effectors appear to be neurosecretory cells in the *pars intercerebralis*. Details of this inductive process remain obstinately obscure although later chapters (see Chapters 14 and 16) will speculate on the events involved. However, several *formal* models for the photoperiodic counter have been proposed. These will be reviewed in the following section.

1. Formal models for photoperiodic summation

Several formal models for photoperiodic summation have been proposed. Goryshin and Tyshchenko (1974) considered that day length (or night length) was 'measured' by an 'oscillator link' (equivalent to the photoperiodic clock) only capable of the qualitative distinction between long nights and short nights. This information was then accumulated in

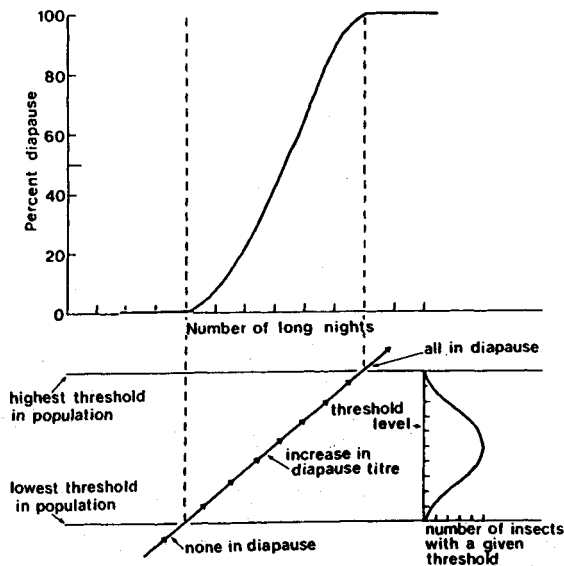


Fig. 12.11. *Sarcophaga argyrostoma*. A theoretical model to account for the summation of short days (long nights) by the photoperiodic counter. *Upper panel*: the increase in pupal diapause with an increasing number of long nights experienced during the larval sensitive period. *Lower panel*: the theoretical increase in a "diapause titre" with an increasing number of inductive long nights, plotted against the presumed lowest and highest individual larval thresholds in the population. As more and more larvae cross their individual thresholds a greater proportion enters diapause. *At right*: the theoretical distribution of diapause thresholds in the population. (After Gibbs, 1975).

discrete 'information packets' by a thermostable 'memory link' (equivalent to the counter) to some sort of internal threshold. This threshold then controlled the third stage or 'neuroendocrine link' (effector mechanism) which directed development down the two alternate pathways, diapause or nondiapause. The exact location of these 'links' was not determined, but the *pars intercerebralis* of the brain was a favoured site (see Chapter 14).

Gibbs (1975) proposed a rather similar model for long night summation in the flesh fly, *Sarcophaga argyrostoma* (Fig. 12.11). In this species, the summation of short-day (or long-night) cycles was seen as the accumulation of an unknown substance or 'diapause titre' which prevented the release of an ecdysiotropic signal from the pupal brain. The stored information was compared with an internal and presumably inherited threshold value: if it exceeded this threshold the pupa entered diapause, if not, it developed. The model also incorporated a distribution of threshold values in the population that was reflected by an increasing proportion of the insects entering diapause as the number of long nights rose. As more individuals crossed their particular internal thresholds, the proportion of the population entering diapause also rose. Both of these models incorporated the same cybernetic ideas: a 'clock' that provided an all-or-nothing ('yes-or-no') control of the diapause or nondiapause state, a temperature-compensated 'counter' mechanism that accumulated such information in quantal 'packets', and a downstream endocrine effector.

More recent models for the photoperiodic counter (Vaz Nunes and Veerman, 1982; Vaz Nunes, 1990) developed the ideas put forward by Gibbs (1975). Mathematical models were generated, testable by computer simulation, that involved temperature effects on the accumulation of long and short nights in quantal packets, to an internal 'induction sum'. Such ideas for the clock-counter mechanism will be considered further in Chapter 13.

In most insects the sensitive stage and the resulting diapause occur in the same individual, although often in different instars. Therefore, although there may be considerable internal reorganisation of tissues and organs during metamorphosis, there is continuity of the central nervous system between the two. Accumulation of photoperiodic information and its transmission to the endocrine effectors, therefore, most likely occurs within the CNS or brain. This continuity, however, is not essential: in species such as the parasitic wasp *Nasonia vitripennis* and the blow fly *Calliphora vicina*, for example, photoperiodic 'information' is transmitted *via* the undifferentiated egg from mother to larva. In these species the brain and CNS cannot be involved in such transmission. There are at least three ways in which this might be achieved. (1) 'Product accumulation' may occur under either long or short day length, or both, and the product is incorporated into the ovarian egg as a cytoplasmic factor. (2) 'Product accumulation' results in a substance operating a genetic 'switch' mechanism in the oöcyte nucleus. Or (3) there is simply a transfer of circadian phase from the mother to the larva via the egg: this, for example, seems to occur in the Queensland fruit fly, *Dacus tryoni* (Bateman, 1955). The first two seem most likely in view of the strong evidence for some sort of product accumulation. There is, however, little to enable a choice to be made between them, unless the probable 'dilution' of a cytoplasmic factor during morphogenesis rules out the first.

ANNOTATED SUMMARY

1. Superimposed on the photoperiodic clock is a mechanism called the photoperiodic 'counter' that serves to accumulate successive long nights (short days) or short nights (long days) to a point at which diapause or diapause-free developmental pathways are determined.

2. Both long nights and short nights may be accumulated, although the effects of the latter may be physiologically 'stronger'.
3. In several species the accumulation of photoperiodic cycles during the sensitive period is a temperature compensated process.
4. Induction of diapause or a seasonally appropriate morph is frequently effected by an interaction between the length of the *sensitive period* (SP) and the *number* of long or short nights needed to produce a 50 per cent response (the *required day number*, or RDN). These two components have different temperature coefficients, the SP being temperature sensitive, but the RDN having a high degree of temperature compensation. Insects raised at high temperature, therefore, may reach the end of the sensitive period before 'seeing' a sufficient *number* of inductive cycles. At a lower temperature, on the other hand, they may see a sufficient number of inductive cycles before the end of the SP and the alternate pathway is followed.
5. In at least three species (the aphids *Megoura viciae* and *Aphis fabae*, and the flesh fly *Sarcophaga argyrostoma*) accumulation of long nights is temperature compensated, whereas accumulation of short nights is not. This observation forms the basis of the 'double circadian oscillator' model for the clock-counter mechanism (see Chapter 13).
6. In the mosquito *Aedes atropalpus*, and a number of other species, the required day number varies with latitude, northerly populations requiring fewer long nights to complete the diapause programme than populations to the south.
7. Aspects of feeding, either quantitative or qualitative, may also affect photoperiodic induction, often by altering the sensitive period without affecting the operation of the counter.
8. Manipulating the length of the sensitive period by factors other than temperature may alter the proportion of insects entering diapause. For example, shortening the larval sensitive period in *S. argyrostoma* by overcrowding, or by premature extraction of larvae from their food, may result in early pupariation and a reduction of pupal diapause. Lengthening the sensitive period by temporary starvation of young larvae, on the other hand, may result in an increase in diapause.
9. Formal models for photoperiodic summation incorporate the temperature compensated accumulation of an unknown 'diapause titre' or 'induction sum' to an internal threshold that determines diapause or nondiapause development. Thresholds in individual insects show a normal distribution in the population, the incidence of diapause rising as more and more individuals cross their internal thresholds.

CHAPTER 13

PHOTOPERIODIC TIME MEASUREMENT: THE CLOCK-COUNTER MECHANISM

Ideas are like rabbits. You get a couple and learn how to handle them, and pretty soon you have a dozen.
John Steinbeck

CONTENTS

Introduction	396
A. <i>Models for Photoperiodic Induction</i>	396
1. Hourglass models	396
2. A non-clock role for the circadian system	398
(a) The 'resonance' effect	398
(b) The hourglass timer - oscillator counter model	398
3. A clock role for the circadian system	399
(a) Bünning's hypothesis and 'external coincidence'	399
(b) External coincidence: the damped oscillator model	400
(c) External coincidence and the multioscillator clock	401
(d) Internal coincidence and the multioscillator clock	401
(e) Amplitude models	402
4. Quantitative clock models	403
(a) The clock-commander model	403
(b) The double circadian oscillator model	403
B. <i>Hourglass-like or Circadian Timers?</i>	404
C. <i>Internal or External Coincidence: How Many Oscillators are Involved?</i>	405
D. <i>Can Overt Rhythms be Used as 'Hands of the Clock'?</i>	414
1. Photoperiodic induction in <i>Pectinophora gossypiella</i>	414
(a) Chilling and the use of concurrent temperature cycles	415
(b) Entrainment and induction when T is close to τ	415
(c) Selection for 'early' and 'late' eclosion strains in <i>Pectinophora gossypiella</i> and its effect on induction	417
2. Photoperiodic induction in <i>Sarcophaga argyrostoma</i>	418
(a) Similarities between eclosion and diapause induction: 'hands' of the clock	418
(b) Use of eclosion phase response curves for calculating photoperiodic phase relations	419
(c) Parallels between entrainment and diapause induction in 'complete' and 'skeleton' photoperiods (T = 24 hours)	420
(d) Entrainment and induction in cycles where T is not equal to 24 hours: the 'T experiment'	422
(e) Asymmetrical skeletons, or night interruptions when T is not equal to τ	424

3. Overt rhythms as 'hands of the clock'	425
E. <i>What is the Evidence for a Specific Photoinducible Phase?</i>	427
F. <i>Current Status of the Models</i>	428
G. Annotated Summary	429

INTRODUCTION

THIS chapter reviews formal or abstract models proposed for photoperiodic time measurement (PPTM). They are the outcome of an immense body of experimental work carried out on a wide range of insects showing different types of response (diapause, seasonal morphs, long-day, short-day etc) and subjected to an equally diverse array of experimental protocols generally involving manipulation of the light cycle.

According to Pavlidis (1981) a model is a hypothesis about how a physical system works; it must summarise available data in a concise fashion, and predict behaviour of the system in new circumstances (i.e. it must be testable). Properly designed tests may reveal deficiencies in current knowledge suggesting new experiments or better models. A 'good' model may also act as a guide to the 'concrete' biochemical processes involved.

Writing this chapter has been governed by four guiding principles: (1) That the circadian system is *somehow* involved in photoperiodic time measurement; i.e. Bünning's general hypothesis is basically correct (Chapter 11). (2) That PPTM involves a number of components, reflecting the multioscillator circadian system (Chapter 6). (3) That at least some of these oscillators are damping, in extreme cases leading to hourglass-like properties (Chapter 11). And (4) that PPTM is a two-stage process involving night length measurement by the 'clock' and accumulation of successive night lengths by the 'counter' (Chapter 12). Particular attention will be paid to 'external' and 'internal coincidence', the 'hourglass timer-oscillator counter' model, and the 'double circadian oscillator model', all of which are based on sound experimental data, have strong predictive value, and have been extensively tested, both experimentally and by computer simulations.

A. MODELS FOR PHOTOPERIODIC INDUCTION

Models for the photoperiodic clock have been reviewed by Takeda and Skopik (1997) and Vaz Nunes and Saunders (1999), the latter forming the basis for much of the present chapter. Table 13.1 presents most of the more significant models for seasonal time measurement (PPTM), from early ideas of a night length hourglass (non-repetitive) to more recent multioscillator (circadian based) timers incorporating damping systems and the simultaneous operation of the 'counter'. This array of models is not merely a list compiled by investigators over time. It represents an 'evolutionary' development of ideas regarding photoperiodic time measurement, becoming increasingly more plausible by the incorporation of experimental data and considerations of circadian biology. A brief description of these models will be given here; the original papers should be consulted for greater details.

1. Hourglass models

Hourglass clocks, usually measuring night length, have been proposed on a number of occasions, but the strongest experimental evidence for such a non-repetitive timer (model 1) comes from the extensive work of A. D. Lees on the green vetch aphid, *Megoura viciae* (Lees, 1965, 1966a, 1967a, 1973, 1981, 1984, 1986). In this insect reproductive mode is regulated by

TABLE 13.1. Models for the photoperiodic clock in insects (Vaz Nunes and Saunders, 1999).

Clock models			
A. Hourglass clocks	Circadian system has no influence on photoperiodic time measurement	Model 1	Lees (1973)
B. A non-clock role for the circadian system			
1.	The resonance effect. Magnitude of the photoperiodic response depends on circadian system's proximity to resonance (i.e. when T is close to τ)	Model 2	Pittendrigh (1972)
2.	The hourglass timer-oscillator counter model. The clock can be an hourglass or a circadian oscillator, but the circadian system affects the counter.	Model 3	Vaz Nunes & Veerman (1982)
C. A clock role for the circadian system			
1.	External coincidence. Photoperiodic induction (e.g. diapause) occurs only when a photoinducible phase (ϕ_i) of the circadian system coincides with darkness ^a :		Bünning (1936)
a.	ϕ_i is a phase of a self-sustained pacemaker	Model 4	Pittendrigh (1966)
b.	ϕ_i is a phase of a damping pacemaker	Model 5	Lewis & Saunders (1987)
c.	ϕ_i is a phase of a slave oscillation	Model 6	Vaz Nunes et al. (1991a,b)
2.	Internal coincidence. Photoperiodic induction occurs only when critical phases of two separate oscillators (x and y), internal to the circadian system, coincide in time:		Pittendrigh (1972)
a.	x and y are mutually coupled oscillators in a complex pacemaker	Model 7	Tyshchenko (1966)
b.	x and y are separate (uncoupled) light-entrainable oscillators	Model 8	Pittendrigh et al. (1984)
c.	x and y are pacemaker and slave	Model 9	Beck (1985b)
d.	x and y are both slaves	Model 10	Pittendrigh (1981b)
3.	Amplitude model. The clock is a circadian oscillator whose amplitude is temperature dependent.	Model 11	Pittendrigh et al. (1991)
D. Quantitative clock models			
1.	The clock-commander model. The clock measures night length in a quantitative manner; the commander (comparable to the counter) determines whether night is long or short.	Model 12	Zaslavskii (1988)
2.	The double circadian oscillator model. The clock consists of two damping circadian oscillators, each of which measures night length and gives it a zero or positive quantitative value. The critical night length is determined by the counter.	Model 13	Vaz Nunes (1998)
a.	In the original version (Pittendrigh, 1966) induction of development occurred when ϕ_i coincided with light.		

night length, short nights of summer leading to parthenogenetic and viviparous morphs (virginoparae), whereas the long nights of autumn induce the production of egg-laying, sexual forms, or oviparae.

The model for night-length measurement was seen by Lees as a linked sequence of four biochemical reactions distinguished on the basis of their responses to light breaks in the dark component of the cycle (see Chapter 11, section C for the experimental basis of this model). (1) During the first 2 - 3 hours of the night, the timing reaction was reversed by blue light. After a short light pulse at this stage resetting of the timing mechanism occurred and night-length measurement restarted after the light pulse. (2) From the 3rd to the 4th hour of the night the system became insensitive to short light pulses. (3) Between the 5th hour and just short of the critical night length the system was again photosensitive and responded to a broad spectrum of light; its virginopara-promotive effect was irreversible by subsequent exposure to a dark period of more than the critical length. (4) This stage began at the critical night length of 9.5 h and caused an ovipara-promotive effect. In natural photoperiods, either stage 3 was illuminated, whereupon virginoparae were produced, or lights-on occurred in stage 4, resulting in oviparae. After each measured night, a period of illumination of about 4 - 6 h was needed to 'prime' the hourglass for its next act of night-length measurement.

This simple non-oscillatory night length timer for *M. viciae* was the outcome of years of investigation and uniformly 'negative' responses to protocols such as Nanda-Hamner, Bünsow and bistability experiments which had produced evidence for a circadian involvement in other species (see Chapter 11). In Lees's extensive study no evidence for circadian rhythmicity was ever obtained. However, later experiments by Vaz Nunes and Hardie (1993) showed that this hitherto 'classical' example of an hourglass timer may also have its basis in the circadian system (see below).

2. A non-clock role for the circadian system

(a) The 'resonance' effect

C. S. Pittendrigh introduced the 'resonance' principle (model 2) in 1972. He suggested that circadian oscillators were *not necessarily* involved in time measurement *per se* (i.e. as part of the clock). However, the *consequences* of time measurement - perhaps the accumulating effects of long and short nights by the counter - might be affected by interactions between the environmental light cycle and the circadian system. These interactions could explain 'positive' Nanda-Hamner profiles (see Chapter 11) in which the inductive effect was high in cycles where the light (L) and the dark (D) components were of a cycle length (T) which was close to the natural period (τ) of the circadian system, or multiples thereof (i.e. τ or modulo τ), but was low when T was far from τ . In other words, photoperiodic induction (which may be by *any* sort of clock) was most effective when the circadian system was at 'resonance' or in harmony with the photic environment, and least effective when it was constantly being subjected to out-of-phase light signals.

(b) The hourglass timer - oscillator counter model

The 'hourglass timer - oscillator counter' model (model 3) (Vaz Nunes and Veerman, 1982) is an explicit version of this non-clock role for the circadian system, originally proposed for diapause induction in the two-spotted spider mite, *Tetranychus urticae*. The model proposed that the length of the night was measured by an hourglass-like clock, but 'resonance'

affected the counter mechanism accumulating the covert effects of successive long or short nights. The hourglass timer was a computerised version of the *Megoura* clock and was able to generate similar photoperiodic responses to those obtained experimentally with Lees's protocols performed with the mite (Vaz Nunes and Veerman, 1986a). However, in the mite the positions of the 'peaks' and 'troughs' observed in Nanda-Hamner experiments were not related to the total cycle length (T) as suggested by Pittendrigh (1972), but rather to scotophase duration (D). Peaks occurred when $D = 12 + n\tau$ and troughs when $D = 24 + n\tau$ and *not* when $T = n\tau$ and $T = \frac{1}{2}\tau + n\tau$ (Vaz Nunes and Veerman, 1986b). This, however, comes about because the circadian oscillation(s) involved are 'damped out' in protracted periods of light (see below).

3. A clock role for the circadian system

(a) Bünning's hypothesis and 'external coincidence'

In 1936, E. Bünning proposed that time measurement in photoperiodic induction was dependent on the 'endogenous diurnal rhythm' (= circadian rhythm) then known to provide temporal organisation in plants. In an explicit model, already introduced in Chapter 11, he proposed that the 24-h cycle comprised two half-cycles differing in their sensitivity to light. The first 12 hours constituted the 'photophil' (= subjective day) and the second 12 hours the 'scotophil' (= subjective night). Short-day (or long-night) effects were produced when the light was restricted to the photophil half-cycle, but long-day (or short-night) effects were produced when light extended into the scotophil half-cycle. Light therefore had a *dual role*: entrainment of the circadian rhythm and photoinduction (see Fig. 11.1).

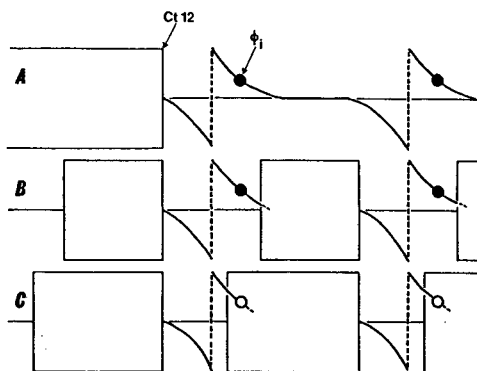


Fig. 13.1. Pittendrigh's 'external coincidence' model for the photoperiodic clock showing action of the photoperiodic oscillator, represented as a Type 0 phase response curve. A – transfer from LL (open box) to darkness; B – under short days or long nights (LD 12:12); C – under long days or short nights (LD 16:8). The phase of the oscillator at the end of an extended period of light is Ct 12 in all cases; the photoinducible phase (ϕ_i) lies about 9.5 hours later. In continuous darkness (A) or under long nights (B) ϕ_i lies in the dark leading to a high incidence of diapause; under short nights (C) ϕ_i is illuminated, leading to diapause avoidance.

The postulated dual role of light in photoperiodism was further elaborated by Pittendrigh and Minis (1964) in their 'external coincidence model' (model 4), developed from Bünning's

hypothesis, but based on a fuller understanding of the entrainment phenomenon, exemplified by pupal eclosion in *Drosophila pseudoobscura* (Pittendrigh, 1966). The phase of photo-sensitivity was now reduced from a full half-cycle to a much more restricted 'photoinducible phase' (ϕ_i) thought to lie in the latter half of the subjective night (Fig. 13.1). The interactions of the entraining and inducing effects of light in a wide range of simple and complex light regimes were described in a number of papers (Pittendrigh, 1981b, Pittendrigh and Daan, 1976, Pittendrigh and Minis, 1971, 1972, Pittendrigh et al., 1970). Although in the original model induction of *development* was supposed to occur when ϕ_i coincided with *light*, later the emphasis changed, and induction of *diapause* was supposed to occur when ϕ_i coincided with *darkness*. This model proved particularly useful as a working hypothesis for pupal diapause induction in the flesh fly *Sarcophaga argyrostoma* (Saunders, 1976, 1978a, 1979a). The effects of night interruptions, in particular, turned out to be very similar to those in the *Megoura* hourglass (Saunders, 1979a, 1981a). According to the external coincidence model, night-length measurement in 24-h cycles was accomplished by a similar principle: as nights got shorter, the dawn transition of the daily photophase extended 'backwards', eventually to illuminate ϕ_i in the late subjective night. The photo-inducible phase, therefore, was equivalent to Lees's stage 3.

(b) *External coincidence: the damped circadian oscillator model*

External coincidence is a simple model that explains many photoperiodic data (Saunders, 1981c, 1982a). In its original form, however, it predicts a high incidence of diapause in continuous darkness and ultra-long scotophases (Saunders, 1982b) because, in both types of regimes, ϕ_i falls in the dark in each cycle of the oscillation (see Fig. 13.1). In other words, it has limitations in describing the characteristic *shape* of most short-night photoperiodic responses with a drop in diapause incidence toward the left-hand side of the response curve. A modification of the original external coincidence model has been proposed to meet that particular problem. Following a suggestion of Bünning (1969), it was proposed that the oscillator(s) making up the clock are not self-sustained, but damp out within a few cycles unless maintained at high amplitude by a train of 'strong' light pulses (model 5) (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b). This model (see also Chapter 11E) was derived from a feedback control systems approach to circadian rhythmicity (Lewis, 1994; Chapter 7), also used successfully to simulate the biochemical feedback loops in the circadian system of the various *period* mutants of *Drosophila melanogaster* (Lewis et al., 1997; see Chapter 4). It was shown that the feedback control model was analogous to the word models of the mechanisms developed from molecular studies of the circadian system in this species (e.g. Lee et al., 1996). Model 5 suggested that changes in the rate of synthesis (SR) of a hypothetical oscillating chemical (*c*) altered the damping coefficient, with high values of SR leading to a self-sustained oscillation. On the other hand, a very low value of SR led to an extremely damped oscillation resembling an hourglass, because it damped within one cycle below a threshold (Thresh). The photoinducible phase, ϕ_i , occurred when *c* declined and crossed Thresh. A rise in temperature increased SR, whereas a reduction in temperature lowered it. Light reduced the value of *c* and prolonged light held *c* down until the light went off whereupon the oscillation started anew. The critical night length, the shape of the photoperiodic response curve, the declining 'amplitude' of successive peaks and temperature effects on Nanda-Hamner and Bünsow responses, could all be explained by systematic changes in SR, temperature, light intensity and other parameters of this model (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b).

(c) *External coincidence and a multioscillator clock*

By further analogy with *Drosophila* eclosion rhythms, Pittendrigh et al. (1984) suggested that the external coincidence clock could be a multioscillator system with a 'pacemaker' and one or more 'slave' oscillators. In that context, ϕ_i could be either a phase of the pacemaker (model 4 and 5) or of a driven 'slave'. Each was theoretically possible, although the latter thought to be more likely. The same idea was also proposed by Saunders (1982b), to explain the decline in diapause incidence in ultra-short nights in the flesh-fly, *Sarcophaga argyrostoma* (see Fig. 10.12). This decline in diapause could not be explained easily by the original external coincidence model. Another shortcoming of the single-oscillator external coincidence models was its inability to explain some results obtained with *T. urticae*, i.e. the fact that the clock was non-repetitive (demonstrated experimentally by Veerman and Vaz Nunes in 1987), whereas Nanda-Hamner experiments - revealing four peaks and troughs of diapause induction - suggested a circadian clock that ran at least three to four cycles before damping out.

An explicit model of the pacemaker-slave version with ϕ_i being a phase of the slave was developed by Vaz Nunes et al. (1991a,b) (model 6) to meet this particular problem. In this *pacemaker-slave* model the pacemaker was a self-sustained or slowly damping, light-entrainable oscillator and the slave a heavily damped, but also light-entrainable oscillator which was coupled to the pacemaker. Strong coupling made the slave - which was the actual clock - a self-sustained or slowly damping circadian clock. Weak coupling, on the other hand, made it a strongly damping, and therefore a non-repetitive clock, indistinguishable from an 'hourglass'. It was suggested further, that the pacemaker could affect the counter in a way similar to the way the circadian system did in the *hourglass timer - oscillator counter* model. According to the pacemaker-slave model, the clock in *T. urticae* could therefore be described as a system with a non-damping pacemaker and a weakly coupled slave, with a circadian effect on the counter by the pacemaker (Vaz Nunes et al., 1991b).

(d) *Internal coincidence and the multioscillator clock*

Evidence that the circadian system of insects and other higher organisms comprises several if not many circadian oscillators (see Chapter 6), led Pittendrigh (1960) to propose that photoperiodic induction was also a product of several oscillators and their internal phase relationships. This concept was later called '*internal coincidence*' (Pittendrigh, 1972) to differentiate it from external coincidence; it suggested that light had a *single* role (entrainment) and not the dual role (entrainment and photoinduction) inherent in the latter. Table 13.1 lists several theoretical versions of internal coincidence. These include: phase relationships between two light-entrainable master oscillators or pacemakers, either coupled in a complex pacemaker (model 7) or independent of each other (model 8); phase relationships between a pacemaker and a slave (model 9); or phase relationships among several slaves driven by the same (or different) pacemaker(s) (model 10).

The simplest form of internal coincidence is the 'dawn' and 'dusk' model of Tyshchenko (1966) (model 7) (see also Danilevskii et al., 1970). This model envisaged two pacemakers (sometimes called 'morning' and 'evening') entrained by dawn and dusk, respectively, whose internal phase relationship changed with the length of the photophase. Induction of diapause or development occurred according to the 'overlap' between particular

phases of the two components (Fig. 13.2). Diapause induction in the parasitic wasp, *Nasonia vitripennis*, is open to interpretation using this model (Saunders, 1974).

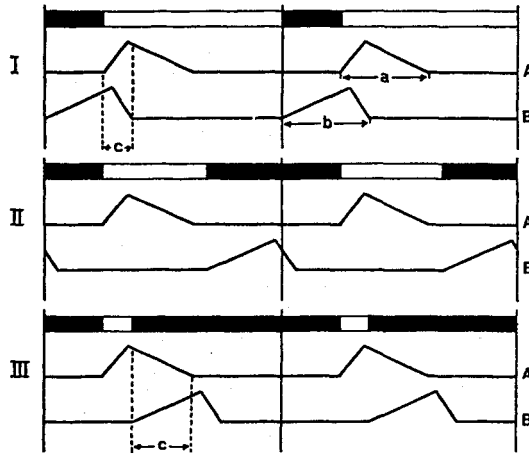


Fig. 13.2. A two-oscillator hypothesis for photoperiodic time measurement ('internal coincidence'; Table 13.1, model 7). I - under long day length temporal coincidence occurs between 'active' phases of the two oscillators. II - under short day lengths temporal coincidence does not occur. III - under ultra-short day lengths the 'active' phases overlap once again. A - oscillator phase-set by dawn; B - oscillator phase-set by dusk; C - area of temporal coincidence. (From Tyshchenko, Reproduced with permission, from 'Biological rhythms in terrestrial arthropods', Ann. Rev. Entomol. 15, 232. Copyright 1970 by Annual Reviews Inc. All rights reserved.)

The 'dual system theory' (DST) of Beck (1985b) was in essence a clock of the pacemaker-slave type (model 9). It consisted of two components, an *S* system beginning at dusk and capable of free running in DD (*i.e.* a circadian oscillator) and a *P* system that free-ran in LL but which was entrained by *S* in darkness and dictated the position of a 'determination gate'. The level of *S* at the time of gate closure then determined the incidence of diapause. The *S* system may be identified as a pacemaker, the *P* system as a slave.

Internal coincidence as a function of mutual phase relationships between several slave oscillations (model 10) was explored by Pittendrigh (1981b). An array of 9 such slaves, each differing in period (τ), damping coefficient (ϵ) and coupling strength (C) to the pacemaker, were shown to change their internal temporal order according to the period of the driving light cycle (T), photoperiod, and temperature. According to Pittendrigh, these changes in the temporal programme could provide a mechanism for night-length measurement as they were capable of yielding an explanation for Nanda-Hamner profiles (see Chapter 11), the shape of the photoperiodic response curve and temperature dependence (or temperature compensation) of the critical night length.

(e) Amplitude models

According to the *amplitude model* developed by Pittendrigh et al. (1991) for eclosion rhythms in *Drosophila*, the amplitude of a circadian oscillator is, in contrast to its period, temperature dependent and also shows latitudinal variation (model 11). It was suggested that this model, which gave an elegant explanation for the effects of temperature and latitude on eclosion rhythms in both *D. auraria* and *D. littoralis*, also might be valid for photoperiodic

time measurement. However, although the amplitude model certainly has appeal, its validity remains to be tested.

4. Quantitative clock models

(a) The 'clock-commander' model

In all models described so far, it was the clock itself that distinguished between 'long' and 'short' nights, relative to a critical length. For example, the coincidence of ϕ_i with either darkness or light determined whether the night was long or short, respectively. It is possible, however, that the clock merely measures the length of a night, whereas the distinction between a long and short night is determined *after* the accumulation of the measured nights has been completed, *i.e.* not by the clock, but by the counter.

Zaslavski's (1988, 1996) 'clock-commander' model (model 12) was based on this hypothesis. The clock's only function was the *quantitative* measurement of night length. The 'commanding' mechanism, in turn, processed this information and determined whether a night was long or short. It was suggested that this commanding mechanism consisted of two antagonistic 'centres', one, the A centre, exhibiting the 'long-day reaction', the other, the I centre, the 'short-day reaction'. Both centres produced their cumulative effect, the total value of which determined whether or not induction (*e.g.* diapause) occurred. In effect, the 'commander' was, therefore, a counter mechanism. Only those nights strong enough (*i.e.* with a high enough inductive value) to result in the cumulative effect exceeding a certain threshold value exerted a long-night effect; weaker nights acted as short nights. It was in this way that the commanding mechanism qualified nights as either long or short. In other words, the critical night length was not a 'fixed' value. This, mostly graphical, model was able to generate a wide range of photoperiodic response curves, including those of the long-day and short-day types (Zaslavski, 1988). However, as the model was based on the natural 24-h cycle only, it cannot be used to simulate non-24-h light-dark cycles.

(b) The double circadian oscillator model

Observations with various insects over the years have indicated that long nights and short nights are more fundamentally different than simply 'long' or 'short'. In particular, it has been observed that the effect of temperature on long nights and short nights is rather different, in that short nights appear much more sensitive to temperature than long nights (Goryshin and Tyshchenko, 1973; Hardie, 1990; Saunders, 1992; Vaz Nunes and Hardie, unpublished)(see Chapter 12). Moreover, in the vetch aphid *M. viciae* and the large cabbage white butterfly, *Pieris brassicae*, long and short nights seemed to be determined in different ways (Vaz Nunes and Hardie, 1993; Vaz Nunes, 1994; Dumortier, 1994). In some cases only long nights were accumulated, not short nights (Goryshin and Tyshchenko, 1973; Saunders, 1981b). These observations were difficult to explain if the difference between a short and a long night was simply the coincidence or non-coincidence of ϕ_i with light, respectively. It seemed that the clock involved in long- and short-night measurement consisted of two different mechanisms. To take these findings into account, Vaz Nunes (1998) proposed the 'double circadian oscillator' model (model 13) in which there are two circadian oscillations, each determining the length of a night, and each giving it a quantitative value which is either zero or positive. One of the mechanisms (the 'long night' or LN system) gave the night a positive value when, at lights-on, the oscillator was in its descending phase (*i.e.* when the night was relatively long).

The other mechanism (the 'short night' or SN system) gave the scotophase a positive value when, at lights-on, the oscillator was in its ascending phase and above a certain threshold (*i.e.* when the night was relatively short). Both oscillating systems were based on the feedback control system developed by Lewis (1994) (see Chapter 7). They were not coupled and each oscillator had its own period (τ), damping rate (SR) and sensitivity to light. This model shared certain features with Zaslavski's clock-commander model: (1) The SN and LN systems could be compared to the A and I centres of Zaslavski's model. However, it should be noted that here the LN and SN systems were part of the clock, not of the 'commander'. (2) Night length was determined in a quantitative way. And (3) there was no 'set' critical night length. Its duration depended entirely on the total cumulative effect of the LN and SN systems in relation to an induction threshold. Like Zaslavski's model, the double oscillator model could generate the shapes of a large variety of photoperiodic response curves, from short-day to long-day shapes. And, in addition to Zaslavski's, it could also generate response curves of various photoperiodic regimes, with both natural and unnatural cycle lengths.

This brief historical overview of the models proposed for photoperiodic time measurement plots an advancing complexity (and hopefully, plausibility) of mechanism as more becomes known about the photoperiodic response and the properties of the circadian system which are thought to underlie it. Starting with simple non-repetitive hourglass-like timers, models progressed through single oscillator systems based upon the entrainment phenomenon, to multiple oscillator models incorporating damping components and the 'counter' principle. The most recent proposition (the 'double circadian oscillator model') is based upon sound experimental data, is fully computer testable, and currently provides a plausible explanation for most features of the photoperiodic response.

In the remaining parts of this chapter we will address several important questions raised by these models. These include:

1. How different are circadian-based and hourglass-like timers?
2. What is the evidence for multiple oscillators?
3. Can overt rhythms be used as 'hands' of the photoperiodic clock?
4. Is there a specific light-sensitive (photoinducible) phase, as in the external coincidence model?
5. What do photoperiodic clock models tell us?

B. HOURGLASS-LIKE OR CIRCADIAN TIMERS?

For several decades, the study of photoperiodic time measurement (PPTM) was split by opposing views. Some authors, notably Lees (1973), held that night length measurement was accomplished by a non-oscillatory or hourglass-like mechanism, whereas others, notably Bünning (1936, 1960) and Pittendrigh (1972) held an alternative view that the circadian system somehow provided the necessary time measurement. Crucial in this discussion was the outcome of experiments such as Nanda-Hamner and Bünsow protocols, and those specifically designed to test for circadian involvement, such as the T-experiment or the bistability response. In some cases these experiments indicated a circadian basis for PPTM, in others an hourglass-like timer. The outcome of these experiments is reviewed in Chapter 11, along with evidence that the apparently opposing views are not so distinct. In short, 'positive' and 'negative' responses to these experiments may arise because of the expression (or not) of an underlying

circadian component. 'Negative' or hourglass-like responses, for example, may occur because the circadian oscillations involved in PPTM are heavily damping (Lewis and Saunders, 1987; Saunders and Lewis, 1987a, b), so that night length measurement occurs only once in an extended 'night' rather than repetitively.

C. INTERNAL AND EXTERNAL COINCIDENCE: HOW MANY OSCILLATORS ARE INVOLVED?

Two of the more influential models – 'external coincidence' (Table 13.1, models 4 to 6) and 'internal coincidence' (models 7 to 10) – were introduced by Pittendrigh (1972, and earlier papers). When formulating these explicit versions of Bünning's general hypothesis, Pittendrigh drew attention to two important differences between them. The original version of external coincidence was seen to consist of a *single* oscillator which, like the circadian pacemaker regulating the *Drosophila pseudoobscura* eclosion rhythm, was reset by light pulses in excess of about 12 hours to a narrow range of phases. Short night induction of development was brought about by a temporal coincidence between a particular light sensitive or *photoinducible phase* (ϕ_i) and the dawn transition of the photoperiod, whereas long night induction of diapause occurred when ϕ_i fell in darkness. In contrast, the internal coincidence model was thought to contain (at least) *two* oscillators, one phase set by dawn and the other by dusk, whose mutual phase relationship changed as the photophase either shortened or lengthened with season. The models thus differed on two counts: (1) the number of oscillators involved, and (2) the existence (in external coincidence) of a photoinducible phase. Tests for external and internal coincidence and the number of circadian oscillators involved in photoperiodic time measurement will be considered here. Evidence for a light sensitive phase will be examined in section E.

Although a number of authors have interpreted experimental data in terms of these models, very few attempts have been made to distinguish between them. Protocols to do this have included transfer of insects from a train of light cycles (LD) to continuous darkness (DD), the use of temperature cycles (thermoperiods) in DD, and the use of ultra-short photophases. Results of these procedures have produced data that may be *consistent with* (either of) the models, but none has provided *unequivocal* discrimination between them.

Transfer from LD to DD. Diapause induction in the parasitic wasp *Nasonia vitripennis* and its flesh fly host, *Sarcophaga argyrostoma*, was investigated in experiments involving the transfer of insects from LD to DD (Saunders 1978a). Newly emerged females of *N. vitripennis* were exposed to five consecutive cycles of long nights (LD 12:12) or short nights (LD 16:8) before being transferred to darkness; control groups were maintained throughout in LD 12:12, LD 16:8, or DD. Host puparia (*S. argyrostoma*) were replaced daily. Since photoperiodic induction is maternal in this species (Saunders, 1966a) this procedure provided a daily monitor of the type of progeny (diapause or non-diapause) produced by each female. Results (Fig. 13.3) showed that wasps experiencing five long nights and then darkness switched one by one to the production of diapausing larvae in a manner similar to those experiencing long nights throughout their adult life. The DD control showed no upward trend and never exceeded 40 per cent diapause. Wasps exposed to five *short*-night cycles before DD, on the other hand, continued to produce nondiapausing broods (like the LD 16:8 control) for a further 12 days after transfer to darkness. These results showed that five long or short nights were sufficient to 'programme' the wasps for a complete switch to the production of diapause or nondiapause

broods, respectively, despite the fact that the light cycles were discontinued before any diapause broods appeared.

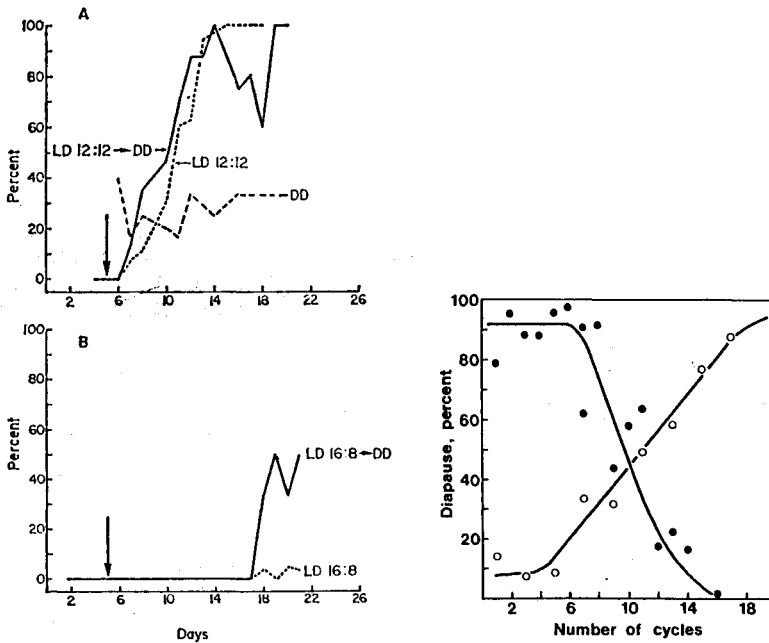


Fig. 13.3 (Left). Transfer of adult females of *Nasonia vitripennis* from LD to DD and its effect on diapause induction. A - release from LD 12:12 to DD after five light cycles; B - release from LD 16:8 to DD after five light cycles. Note that (in A) the rate of switching to diapause larva production continues to rise after transfer to DD in the same manner as in those females maintained in short days throughout. The DD control, on the other hand, is erratic and lacks a clear upward trend. This behaviour may be attributed to putative separate 'dawn' and 'dusk' oscillators of the photoperiodic clock free-running in DD, but maintaining their mutual phase relationship as entrained in LD 12:12, and hence causing diapause induction. Conversely, exposure to five cycles of LD 16:8 (in B) 'programme' the insects for a long period in which they produce no diapause larvae (after Saunders, 1978b).

Fig. 13.4 (Right). *Sarcophaga argyrostoma*. Diapause incidence in cultures transferred from LD 12:12 as embryos into an increasing number of short nights (LD 18:6), and then darkness (closed circles); or transferred from LL as embryos into an increasing number of long nights (LD 12:12), and then darkness (open circles). About 10 to 11 cycles of long or short night are required to reach 50 per cent diapause. (From Saunders, 1980b.)

In comparable experiments with *S. argyrostoma* (Fig. 13.4) larval cultures derived from either long-night (LD 12:12) or short-night (LD 16:8) 'conditioned' adults, were exposed to an increasing number of either short- or long-nights before being allowed to complete their development in darkness (Saunders, 1978a, 1992). In the first set the larvae reverted from diapause to nondiapause, and in the second from nondiapause to diapause. Unlike the experiment with *N. vitripennis* there was no evidence that the discontinuation of the light-cycle at any point left the larvae to maintain their trend toward the diapause or nondiapause state. In contrast, it appeared that the effects of short- or long-nights were accumulated as discrete events during the larval sensitive period, with little evidence for a persistent or free-running mechanism. The number of short- or long-nights required to bring the cultures to 50 per cent

diapause (the required day number; see Chapter 12, A) was about 10 to 11 each case. The patterns of switching to diapause or nondiapause provide some circumstantial evidence that the photoperiodic clock differs between the two species. That in *N. vitripennis* resembles internal coincidence, whereas that in *S. argyrostoma* could be interpreted as being of the external type.

The use of thermoperiods. In the second type of experiment the insects were subjected to temperature cycles in the absence of light (see also Chapter 10, A). Females of *N. vitripennis* were raised from the egg stage in DD and then exposed to daily *thermoperiods* ($T = 24$ hours) consisting of different ratios of a higher temperature (23°C) to a lower temperature (13°C) (Saunders, 1973a). It was found that females exposed to fifteen daily thermoperiodic cycles with less than about 13 hours at the higher temperature produced nearly all of their offspring as diapausing larvae whereas those exposed to thermoperiods with a more protracted period at 23°C produced none (see Fig. 10.7). This result showed that a daily thermoperiod could simulate the effects of a photoperiod in diapause control, and demonstrated that light was not *required* for the operation of the metabolic switch. Since temperature cycles and pulses are known to entrain and phase-set circadian rhythms as effectively as light-pulses and cycles (Zimmerman et al., 1968; Chapter 3) it was concluded that thermoperiods were entraining constituent rhythms in a manner comparable to light. Consequently any model for the *Nasonia* clock in which light plays a *direct* role in induction (e.g. the original form of external coincidence) could be ruled out - although a similar clock with a specific light *or* temperature-sensitive phase, as indicated for *Pieris brassicae* by Dumortier and Brunnarius (1977b) (Fig. 13.5), is not excluded. Nevertheless, combining this evidence with that obtained for *N. vitripennis* in resonance experiments (Chapter 11; Fig. 11.9) does suggest that the clock in this species might be of the internal type involving dawn and dusk oscillators independently phase-set by either light *or* temperature. Clearly an unequivocal demonstration of the distinction between these two clock models is still lacking, however.

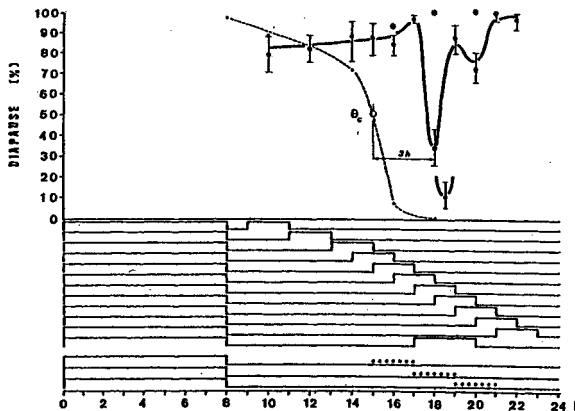


Fig. 13.5. Asymmetrical skeletons (or night interruptions) in *Pieris brassicae* using cycles of temperature in continuous darkness (DD). Cultures were raised in thermoperiodic cycles of 8 hours at 21°C and 16 hours at 13°C , with the 16-hour cold phase systematically interrupted by a supplementary high-temperature pulse of 2 hours at 21°C . The point of maximum sensitivity to the high-temperature pulse occurs about 3 hours after the critical thermoperiod. Dotted curve - critical thermoperiod, θ_c . (From Dumortier and Brunnarius, 1977b.)

Strictly comparable thermoperiodic experiments with *S. argyrostoma* are not feasible because the feeding larvae live in or under a piece of meat that prevents the use of sharply defined temperature cycles. Nevertheless, cultures were raised at 18°C and in long-nights (LD 8:16) until the mature larvae left the meat (8 days), and the 'wandering' larvae were then exposed to daily temperature cycles (23/15°C) in DD until puparium formation (Saunders, 1978b). Control cultures were moved from LD 8:16 to DD at 15° and 23°C, respectively, after a similar period of 8 days. Since 8 long nights are insufficient to raise the proportion of diapause pupae to 'saturation' (Fig. 13.6), the subsequent short or long thermoperiods might be expected to raise or lower the response, if temperature cycles, in the absence of light, were in themselves inductive. Results showed that the incidence of pupal diapause was greater in these cultures exposed to a short thermoperiod and less in those exposed to a long thermoperiod. It was also greater in cultures transferred to 15°C and less in those transferred to 23°C. However, a plot of diapause incidence as a function of the length of the sensitive period (which in turn is a function of temperature) clearly indicated a direct temperature effect. Results for all groups except the overall control (18°C, LD 8:16 throughout) lie close to a straight line, indicating that the proportion of pupae entering diapause is simply a function of the length of the larval developmental period, and therefore of the mean temperature and not thermoperiod (Fig. 13.6). For example, if a 'true' thermoperiodic effect were operating (e.g. one in which temperature cycles were inductive) the datum point for a short thermoperiod (e) ought to be substantially higher, nearer that for a short photoperiod (a), whereas that for a long thermoperiod (f) should be lower. Since (e) and (f) both lie close to the calculated line, the difference between the two can be attributed to a 'temperature effect' altering the rate of development and acting upon the expression of the clock (as in Saunders, 1971; and Chapter 12), whereas the difference between short and long thermoperiods in *N. vitripennis* (Fig. 10.7) is clearly a true 'thermoperiodic effect', acting upon the clock mechanism itself as an inductive force. A similar conclusion, for the *Sarcophaga* case, was reached in a later paper (Saunders, 1984). Once again, the data suggested that *N. vitripennis* uses a clock of the internal coincidence type, whereas external coincidence is entirely adequate for the *S. argyrostoma* case.

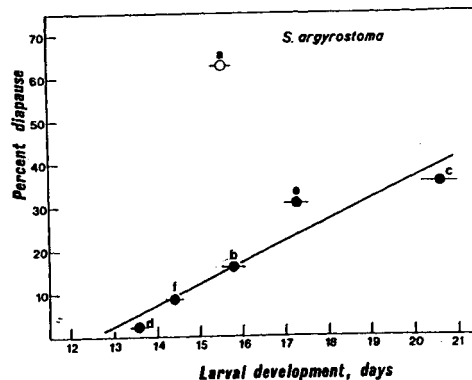


Fig. 13.6. The effect of thermoperiodic cycles on diapause induction in *Sarcophaga argyrostoma*. a - control, LD 8:16, 18°C throughout post-embryonic development; b - LD 8:16, 18° for 8 cycles, then DD at 18°; c - LD 8:16, 18° for 8 cycles, then DD at 15°; d - LD 8:16, 18° for 8 cycles, then DD at 23°; e - LD 8:16, 18° for 8 cycles, then DD with 8 hours at 23° and 16 hours at 15° daily (a 'short' thermoperiod); f - LD 8:16, 18° for 8 cycles, then DD with 16 hours at 23° and 8 hours at 15° daily (a 'long' thermoperiod). The straight line calculated for points b-f shows a linear relationship between diapause incidence and larval duration, suggesting that thermoperiod has no direct effect on diapause induction (see text). (From Saunders, 1978b.)

An internal coincidence type of clock, frequently suggested in the vertebrate literature (e.g. Pittendrigh and Daan, 1976c), is the two-oscillator, morning (M) and evening (E) model. Although this model gains only limited support from the arthropod literature, a parallel is apparent in overt locomotor rhythms of *D. melanogaster* which show clear and apparently independent M and E components (Helfrich-Förster, 2001).

The use of very short photophases. In many insects the incidence of diapause in very short photophases (i.e. less than about 6 hours), and in DD, is less than that in the 'stronger' short photophases (i.e. 8 to 13 hours) (see Chapter 10, A.1). These very short day lengths do not occur in the natural environment (Fig. 10.2), and the responses to them are clearly not a product of natural selection. Nevertheless, these responses must reflect properties of the photoperiodic clock and might also provide important clues to its physiology. As pointed out earlier in this chapter, this drop in diapause incidence cannot be explained easily by a simple one-component clock of the external coincidence type because this model predicts that the photoinducible phase (ϕ_i) must fall in the dark of all short light-pulse regimes, as well as in DD. A study of the phenomenon of entrainment of circadian oscillations to these shorter light-pulses, however, may provide a clue to this drop in diapause incidence. In *Sarcophaga argyrostoma*, for example, pulses shorter than about 4 hours ($240 \mu\text{W cm}^{-2}$) give rise to low-'amplitude' Type 1 phase-response curves, whereas stronger pulses of 5 hours duration or more give rise to high-'amplitude' Type 0 curves (Saunders, 1978a; Winfree, 1970b; see Chapter 3, C.1). Among the known properties of entrainment (Chapter 3, C.2) is the observation that steady state is attained much more rapidly with stronger (Type 0) pulses than with weaker (Type 1) pulses. Examples for the *S. argyrostoma* system exposed to trains of weak (1 and 3 hours) and strong (5 and 12 hours) pulses are depicted in Fig. 13.7. If we now concede that the photoperiodic clock has a multioscillator 'construction' (Saunders, 1978a) it might be argued that night length measurement in strong photophases is more 'precise' than in weak photophases because in the latter the whole system is in a state of internal temporal disorder until it settles down. This argument, which will be developed further, may be particularly relevant to insects like *S. argyrostoma* which have a particular and often circumscribed 'sensitive period' (Chapter 10, B) and a requirement for an effective number of long nights within that period (Chapter 12).

The proposition that the precision of long-night measurement is a function of the speed of attainment of the entrained steady state is open to test provided phase-response curves for particular light-pulses are available (Saunders, 1982b). In *S. argyrostoma*, therefore, this idea may be tested in two ways: (1) using trains of weaker (= short) light-pulses with the first pulse in the train commencing at different phase points or circadian times; and (2) using light-pulses of the same duration but of different light intensity. The rationales and outcomes of these approaches are expanded below.

Experiment 1. A circadian pacemaker exposed to a train of light-pulses reaches a single, unique, steady state regardless of starting phase, but dependent on the period (T) of the driving light-cycle and the strength or duration of the pulse (Chapter 3, C.2). In doing so, however, the pacemaker passes through a series of transient cycles, the number of which and their direction (i.e. advancing or delaying) depending on the phase or circadian time of the initial pulse. In *S. argyrostoma*, for example, the pacemaker governing the rhythm of pupal eclosion, exposed to trains of 4-hour pulses (LD 4:20, T = 24 hours), achieves a steady state in which the pulses come on at Ct 06 and leave the oscillation 4 hours later at Ct 10 (Saunders, 1978a). Populations

in which the initial pulse starts at Ct 15 to Ct 05 attain that steady state *via* a series of advancing transients, varying in number according to the distance of the starting phase from final steady state. In contrast, those starting at Ct 07 to Ct 14 go through a number of delaying transients, again according to initial phase; whereas a population starting at Ct 06 achieves immediate entrainment with no transients. The experimental protocol thus entailed transferring larvae of *S. argyrostoma* from LL to DD (equivalent to Ct 12; see Chapter 3, D), and then exposing them to trains of 4-hour pulses (T 24) with the initial pulse starting at different times after the LL/DD transition in the different experimental groups. Anticipated results would include an *inverse* relationship between diapause incidence and transient number.

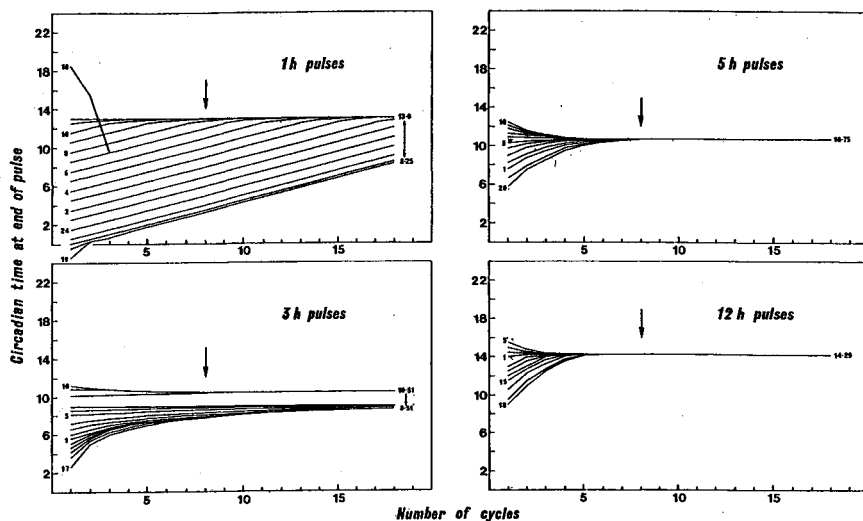


Fig. 13.7. *Sarcophaga argyrostoma*. Computed approaches to steady-state entrainment of the circadian eclosion rhythm to trains of 1-, 3-, 5- and 12-hour pulses of white light ($240 \mu\text{W cm}^{-2}$). The small numbers to the left of the curves show the circadian times at which the first pulse in the train commenced; the curves plot the circadian times at the end of each light-pulse. With 'weak' pulses (1 and 3 hours) approach to steady state is slow; with 'strong' pulses (5 and 12 hours) it is rapid. The vertical arrows (day 8) show the end of larval life at 25°C . (From Saunders, 1978a).

Figure 13.8 shows the results of three such experiments. The small satellite panels of this figure show plots of circadian phases at the onsets of each of the 4-hour light pulses in the train, as computed from the 4-hour-phase response curve (Saunders, 1978a), and for samples commencing at Ct 02, 04, 06 through to Ct 24, respectively. These computed plots show both the sign (advances or delays) and number of transients involved, the latter arbitrarily considered to have subsided when circadian time at the beginning of the pulse was within 0.1 hour of steady state. The central panels of the figure show plots of diapause incidence against computed transient number for experimental populations exposed to eight cycles of LD 4:20 (panel A), twelve cycles of LD 4:20 (B), and to cycles of LD 4:20 throughout larval development (C). Each shows the anticipated inverse relationship between diapause incidence and transient number in which both the regression and the slope are highly significant.

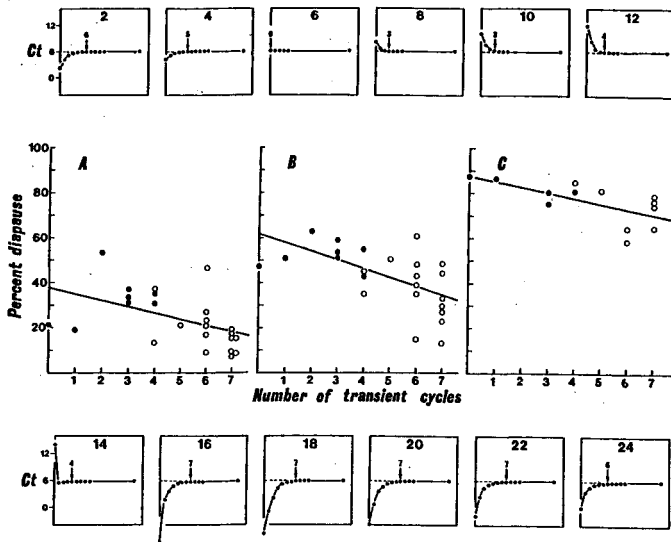


Fig. 13.8. *Sarcophaga argyrostoma*. Diapause incidence and transient number. The twelve satellite panels show the approach to steady-state entrainment in cultures exposed to LD 4:20 but with the first pulse in the train commencing at different circadian times (2, 4, 6, 8, ..., 24). Vertical arrows show when transients are considered to have subsided (arbitrarily to within 0.1 hour of steady state). All cultures achieve an identical steady-state phase relationship in which the 4-hour light-pulse comes on at Ct 6 and leaves it at Ct 10. Different initial conditions, however, result in a different number of transients, and different routes (advancing or delaying) to steady state. Those starting at Ct 16, 18, 20, 22, 24, 2 and 4 go through a series of advancing transients; those starting at Ct 8, 10, 12 and 14 pass through delays. Central panels show the incidence of pupal diapause as a function of transient number: A - cultures exposed to 8 consecutive cycles of LD 4:20, then DD; B - cultures exposed to 12 cycles, then DD; C - cultures exposed to LD 4:20 until puparium formation. Closed circles - delaying transients; open circles - advancing transients. (Saunders, original.)

In a second version of this experiment (Fig. 13.9) cultures of *S. argyrostoma* larvae were exposed to trains of pulses of different duration (1 to 21 hours) with the initial pulses starting at either Ct 24 or at Ct 12. The twelve small satellite plots show that the numbers of transients are small and essentially indistinguishable, for Ct 12- and Ct 24-starts, when strong pulses of greater than 10 hours were used. Computations for shorter pulses, however, show that a greater number of transients are involved. Moreover, those for Ct 24-starts are usually greater in number than those for Ct 12-starts, and advancing rather than delaying. Experimental results (centre panel) show that diapause incidence for 'strong' pulses (>9 or 10 hours) is similar for either Ct 12- or Ct 24-starts, but with 'weaker' pulses, Ct 12-starts produced a consistently higher incidence of diapause than Ct 24-starts. The computed approaches to steady states shown in the small panels show that populations with a Ct 12-start go through delaying transients, whereas those with a Ct 24-start go through advances; in most cases the former also involve fewer transients and a smaller overall shift.

Experiment 2. Since the change from Type 1 to Type 0 response curves (in *S. argyrostoma*) occurs between a 4-hour and a 5-hour pulse with an intensity of $240 \mu\text{W cm}^{-2}$, and a reciprocity between pulse duration and intensity might be expected (Chapter 3, E; Chandrashekeran and Engelmann, 1976) the second experiment involved the use of light-pulses of the same duration,

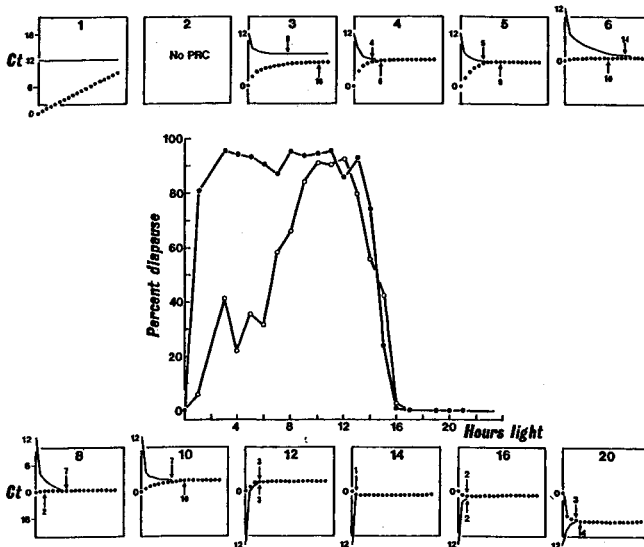


Fig. 13.9. *Sarcophaga argyrostoma*. Diapause incidence in cultures exposed to light-cycles with the first pulse in the train commencing at either Ct 12 or Ct 24/0. The satellite panels show the approaches to steady state for Ct 12-starts and Ct 0-starts for pulse durations of 1-, 3-, 4-, 5-, 6-, 8-, 10-, 12-, 14-, 16- and 20-hour pulses, as computed from eclosion rhythm PRCs. (Ct 12-starts - solid lines; Ct 0 - starts - dotted lines). For pulse strengths of 12 hours or more, steady state is reached rapidly with a minimum of transients. For 'weaker' pulses transient number is higher, particularly (in most cases) for the advancing route (Ct 0-starts). This is reflected in the photoperiodic response curves (central panel) in which diapause incidence is greater for 'weaker' pulses (< 8 hours) when starting at Ct 12 than when starting at Ct 0. (Saunders, original.)

but of an increased irradiance. The anticipated result of this experiment would be that the Type 1 to Type 0 transition should move to shorter pulse durations and, in consequence, shorter pulses of brighter light should induce more diapause than the same pulses of dimmer light. In this experiment larval cultures of *S. argyrostoma* were raised in various photoperiods (LD 2:22, LD 4:20, LD 8:16 and LD 12:12) with the light-phase of the cycle provided either by a 4-W or a 22-W striplight, delivering about $240 \mu\text{W cm}^{-2}$ or about $16,000 \mu\text{W cm}^{-2}$, respectively. Results showed that diapause incidence for the shorter photophases (2 and 4 hours) was considerably enhanced by the brighter light, whereas in the longer pulses (8 and 12 hours) this difference was less marked or absent (Fig. 13.10). One interpretation of this result is that in LD 8:16 and LD 12:12 both bright and dim lights are perceived as 'strong' pulses, presumably because the net radiant exposure has saturated the system. For the shorter pulses, however, the highly significant differences between the two levels of illumination are the result of the shorter pulses becoming effectively 'stronger' in the brighter light, with a more rapid entrainment, fewer transients, and more diapause.

The results of Experiments 1 and 2 may be interpreted in two ways. One is that brighter or longer light pulses increase the rate of entrainment to cycles containing a short photophase. Such a phenomenon becomes important if we remember that a particular number of long nights are required for the induction of diapause (Chapter 12), and that these must be experienced during a restricted 'sensitive period'. If the photoperiodic mechanism is a 'simple' system with one oscillatory component (as in the original external coincidence model), the photoinducible

phase (ϕ_i) would still fall in the dark of each cycle, despite the fact that the pacemaker was passing through a series of transients. If, on the other hand, the clock is a complex, multioscillator, device, with mutual internal phase relationships being important, the number of transients, their magnitude, and possibly their sign, may all become important to the *accuracy* with which night length measurement is effected. These observations seem to indicate, therefore, that the clock contains a number of oscillatory components.

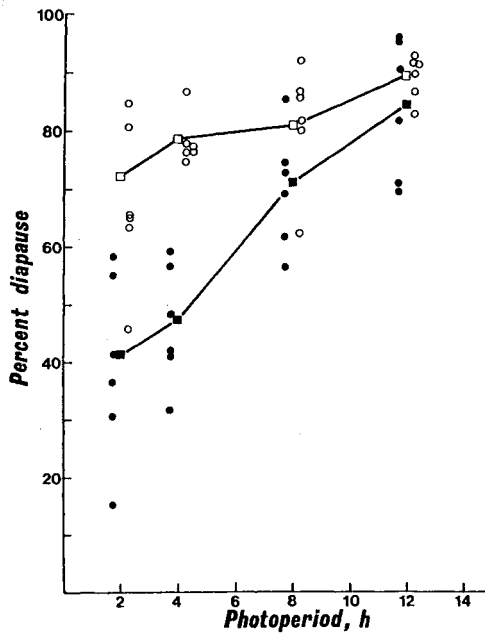


Fig. 13.10. *Sarcophaga argyrostoma*. Diapause incidence in light pulses of different intensity. Closed circles - 240 $\mu\text{W cm}^{-2}$; open circles - 16,000 $\mu\text{W cm}^{-2}$. Closed and open squares - mean values. Diapause incidence is significantly higher in bright light for 2- and 4-hour pulses. (Saunders, original.)

The second interpretation is that brighter or longer light pulses boost damping oscillator(s) to render them more persistent (Lewis and Saunders, 1987), thereby increasing their strength as short days (long nights) in diapause induction. The alternative explanations are not mutually exclusive. Together with the evidence that some form of external coincidence is entirely appropriate for *S. argyrostoma* (see above), these data suggest that the photoperiodic clock in this species consists of a number of circadian oscillators, probably damping in DD, with night length measurement being effected by the coincidence (or not) of light with a specific photoinducible phase. The clock in *N. vitripennis*, on the other hand, seems to resemble that of an internal coincidence device with separate 'dawn' and 'dusk' oscillators. The differences between the two species may indicate diversity in photoperiodic clock mechanisms, which will be addressed below.

D. CAN OVERT RHYTHMS BE USED AS 'HANDS OF THE CLOCK'?

Photoperiodic time measurement is clearly one of the major functions of the circadian system. However, unlike behavioural rhythms (Chapters 2 and 3), there are no overt manifestations of the oscillators involved in photoperiodism. Instead, one has to rely on 'indirect' evidence, using complex experiments such as the Nanda-Hamner and Bünsow protocols (Chapter 11) to reveal the underlying rhythmicity. Largely for this reason early experimenters attempted to use behavioural rhythms as independent measures of period and phase ("hands of the clock") of the presumed photoperiodic oscillators behind the phenomenon, mainly as tests of the external coincidence model. Clearly, the success of this approach depends on the degree of similarity between the overt and covert systems. In some cases it met with considerable success; in others it merely underlined important differences between the two. This section examines the utility of this approach.

1. Photoperiodic induction in *Pectinophora gossypiella*

Using the pink boll worm moth, *Pectinophora gossypiella*, Pittendrigh and Minis (1964, 1971) were among the first to undertake a parallel study of photoperiodic induction and the entrainment of overt rhythms to test the external coincidence model. Three overt rhythms were studied; these were oviposition, egg hatch and pupal eclosion. The supposed phase relationship of ϕ_i to these three rhythms is shown in Fig. 13.11; it is thought to lie late in the subjective night, about 5 hours before ϕ_r for eclosion, a position which corresponds to 'peak B' in night-interruption experiments. This assumption enabled a number of testable predictions to be made about photoperiodic induction in this species. Some of the tests have supported the external coincidence model; others have not.

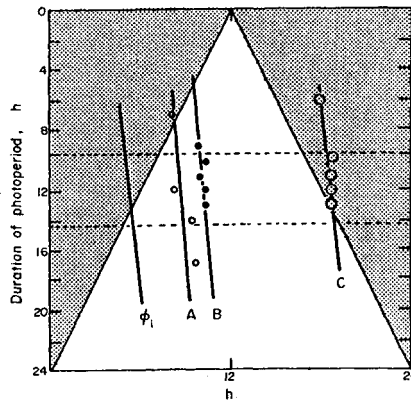


Fig. 13.11. Phase-reference points ($\psi_{R,L}$) for egg hatch (A), pupal eclosion (B) and oviposition (C) rhythms of *Pectinophora gossypiella* as a function of photoperiod. The inducible phase (ϕ_i) is postulated to lie about 5 hours earlier than $\psi_{R,L}$ for the pupal eclosion rhythm. Dashed lines mark the range of natural photoperiods at El Paso, Texas, the source of the strain used. (From Pittendrigh and Minis, 1971.)

(a) Chilling, and the use of concurrent temperature cycles

Pittendrigh and Minis (1971) examined the effects of a light-cycle (LD 8:16) and a concurrent sinusoidal temperature cycle (20° to 29°C) on *P. gossypiella*, the results being assayed simultaneously by diapause induction and the pupal eclosion rhythm. In a cycle of LD 8:16 at constant temperature the peak of eclosion (ϕ_r) occurred soon after dawn (Fig. 13.12). With the addition of the temperature cycle, however, ϕ_r occurred a few hours after the point of lowest temperature. Thus, as the low point of the temperature cycle was displaced to the right (later relative to the light cycle) the phase of the eclosion rhythm was shifted in a similar manner. Since ϕ_i was assumed to occur about 5 hours ahead of ϕ_r for eclosion it, too, would be displaced to the right. Figure 13.12 shows that when ϕ_i was drawn into the light, diapause inhibition occurred. The results therefore were in accord with predictions from the external coincidence model. Similar results were reported for the parasitic wasp *Nasonia vitripennis* (Saunders, 1967, 1968).

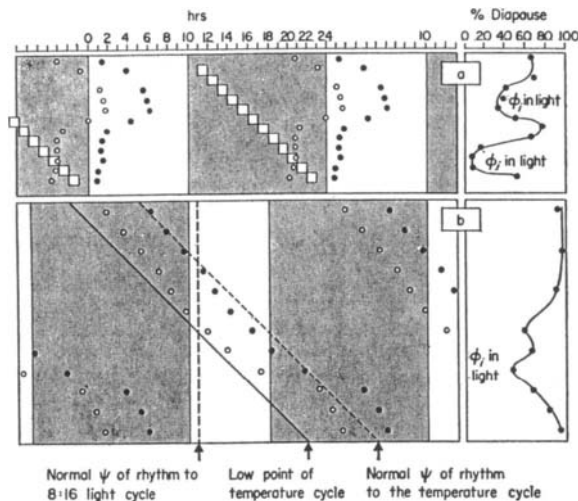


Fig. 13.12. Entrainment and induction by asymmetric skeleton photoperiods in *Pectinophora gossypiella*. Top panel: the phase-reference point of the pupal eclosion rhythm is shown by solid circles; the inducible phase (ϕ_i), 5 hours earlier, by open circles. Panel at right plots the inductive effect (prevention of diapause) of the same skeleton regimes. Lower panel: entrainment and induction by concurrent cycles of light (LD 8:16) and temperature (20 – 29°C , sinusoidal). The position of the rhythm's phase-reference point in LD 8:16 at constant 20°C is indicated by the dashed line 1 hour after dawn. Its position in a sinusoidal temperature regime in constant dark is also indicated by a dashed line 7 hours after the lowest point on the temperature curve. Solid circles mark the rhythm's phase-reference point and open circles mark ϕ_i for the twelve environments characterised by twelve different phase relations between the light and the temperature. Panel at right plots the inductive effect (prevention of diapause) of these same environments. (From Pittendrigh and Minis, 1971.)

(b) Entrainment and induction when T is close to τ

Pittendrigh and Minis (1964, 1971) reported another test of the external coincidence model, again involving the simultaneous assay of circadian phase and diapause induction in *P.*

gossypiella. Earlier studies on the entrainment of the *D. pseudoobscura* system to single light pulses per cycle (Chapter 3) had shown that, within certain limits, the driving oscillation assumed the period (T) of the entraining light-cycle, the discrepancy between τ and T being overcome by a discrete phase-shift. Furthermore, when $T < \tau$ the pulse fell in the late subjective night and caused a phase advance, whereas when $T > \tau$ the pulse fell in the early subjective night to cause a phase delay. Therefore simply by changing the period (T) of the light-cycle the pulse could be made to illuminate different phase-points of the oscillator.

An early application of this test to *P. gossypiella* involved the oviposition rhythm as an assay of phase and the use of single recurrent 15-minute light-pulses to define cycles ranging from $T = 20$ hours 40 minutes to $T = 25$ hours (Pittendrigh and Minis, 1964; Minis, 1965). These values of T encompassed values both greater than and less than τ for this species (22 hours 40 minutes). The prediction was that since the light pulse would illuminate different phase points of the oscillator in different regimes it should coincide with ϕ_i in some, but not in others. However, little difference in the incidence of diapause was observed, and 15 minute pulses were considered, in hindsight, to be inadequate to effect induction.

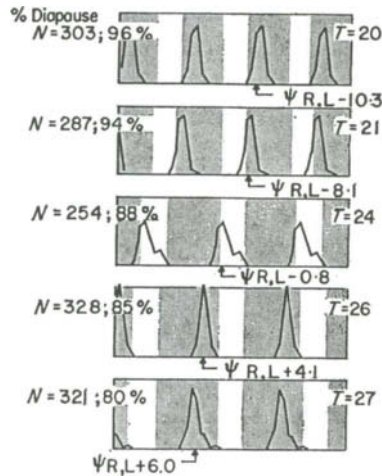


Fig. 13.13. The dependence of induction on T when T is close to τ in *Pectinophora gossypiella*. The entrained steady states of the pupal eclosion rhythm effected by light cycles, all involving an 8-hour photoperiod, whose period (T) ranges from 20 to 27 hours. The photoperiodic induction (measured as per cent diapause) effected by each cycle is indicated on the left (From Pittendrigh and Minis, 1971.)

The experiment was later repeated using light cycles varying from $T = 20$ to $T = 27$ hours, each containing an 8-hour light pulse, and the pupal eclosion rhythm as an independent measure of phase (Pittendrigh and Minis, 1971). Once again, in the shorter cycles ($T = 20$ and $T = 21$ hours) the light-pulses, in steady state, were predicted to fall in the late subjective night, whereas in the longer cycles ($T = 24$, $T = 26$ and $T = 27$ hours) to fall in the early subjective night. Therefore, if ϕ_i occurred about 5 hours ahead of the peak of eclosion it should have been illuminated when $T < 24$ hours but not when $T > 24$ hours, thus causing diapause inhibition in the shorter cycles and diapause promotion in the longer. The results of this experiment (Fig. 13.13) showed that although the phase of the eclosion rhythm assumed a steady state as predicted, the proportion of larvae entering diapause was 80 per cent or over in all light-cycles.

Indeed, although the overall range was small (80 to 96 per cent) the sign of the dependence of diapause incidence on T was the reverse of that expected. Clearly the results provided no clear support for the external coincidence model in *P. gossypiella*.

(c) Selection for 'early' and 'late' eclosion strains in *Pectinophora gossypiella* and its effect on induction

Artificial selection of those adults of *P. gossypiella* which emerged from their pupae earlier or later than the stock culture resulted in two strains ('early' and 'late') which differed in their phase relationship to the light ($\psi_{R,L}$) by about 5 hours (Chapter 3). These strains were used in a test for the external coincidence model (Pittendrigh and Minis, 1971), once again by assuming that ϕ_i occurred some 5 hours ahead of ϕ_r and that a correlated shift in ϕ_i occurred as a consequence of selection. Knowing that the *Pectinophora* system, like that in *D. pseudoobscura*, began from a fixed phase-point at the onset of darkness, the 'late' strain was expected to show less diapause (or more induction of development) under a 12-hour photoperiod, than either 'early' or 'stock'. In other words, the critical day-length for 'late' was predicted to be shorter than that for the other two strains. Theoretically this test had an additional importance. Selection for 'early' and 'late' demonstrated that the phase relationship of the rhythm to the oscillator ($\psi_{R,O}$) had been altered, but not the phase relationship of the oscillator to the light ($\psi_{O,L}$). Consequently, if a change in the photoperiodic response was obtained it might indicate that ϕ_i was part of a driven system or slave; if it was not, it might indicate that ϕ_i was part of a driving oscillator or pacemaker.

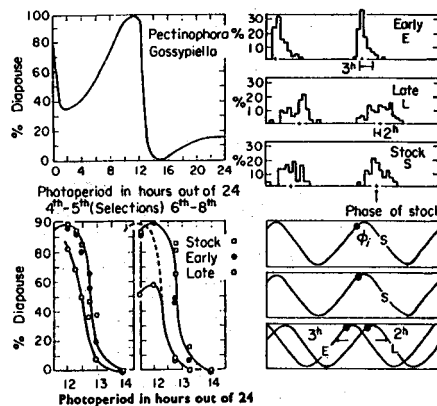


Fig. 13.14. Photoperiodic induction and $\psi_{R,L}$ in *Pectinophora gossypiella*. Upper left: the photoperiodic response curve. Upper right: the effect of eight generations of selection on $\psi_{R,L}$. Lower left: top and middle panels illustrate the coincidence of ϕ_i and light in LD 14:10 and its non-coincidence in LD 12:12; lower panel illustrates the coincidence of ϕ_i and light in LD 12:12 in the 'late' strain on the assumption that $\psi_{R,L}$ of the clock rhythm has also been changed by selection. The dashed line portion of the curve for late (right panel) indicates the normal form of the curve - how it would appear had it merely been displaced. The observed value for LD 11:13 indicates, however, that the curve is depressed and not displaced. (From Pittendrigh and Minis, 1971.)

The results of this experiment (Fig. 13.14) showed that the critical day length was indeed shorter in 'late' than in 'stock' or 'early', after only four to five generations of selection. However, after six to eight generations the response for 'late' also showed that the

photoperiodic responses under strong short day lengths (LD 11:13 and LD 12:12) produced a *lower incidence* of diapause. This may mean that the apparent shift in the critical day length was due to a lowered response to *all* photoperiods (see Chapter 10). Therefore the results of this experiment remained equivocal and provided no clear evidence either for or against the external coincidence model. As Pittendrigh and Minis (1971) pointed out, however, it was of considerable interest that genetic selection for the parameters of one system (the eclosion rhythm) should have such a profound effect upon another (photoperiodic induction).

2. Photoperiodic induction in *Sarcophaga argyrostoma*

The analysis of photoperiodic time measurement in *S. argyrostoma* has passed through three interrelated stages. In the first, reported in Chapter 11, long-period night-interruption (Bünsow) experiments and Nanda-Hamner (resonance) experiments suggested that the circadian system was *somehow* involved in night length measurement (Saunders, 1973b, 1976). In the second stage (Saunders, 1976), the rhythm of pupal eclosion was shown to be sufficiently similar to the photoperiodic oscillator for it to be used as its overt 'hands'. And in the third stage (Saunders, 1978a, 1979a, 1980a, 1981a), data from eclosion rhythm phase response curves were used to compute steady-state phase relationships of the photoinducible phase (ϕ_i) to a variety of simple and complex light regimes. In doing so both the non-parametric entrainment model of Pittendrigh (1965) (see Chapter 3, C) and external coincidence were followed. In every experiment, computed phase relationships of ϕ_i to the light were compared with diapause induction in parallel experiments. Prediction from the model and observation of diapause incidence were extremely close in nearly every case, with high diapause occurring when ϕ_i fell in the dark and low diapause when it fell in the light. Examples of these analyses will be given here.

At the outset, the external coincidence model was adopted for *S. argyrostoma* because it seemed to be a simple 'working hypothesis', open to analysis in terms of entrainment within the circadian system. As the investigation proceeded it became clear that although predictions from the model and experimental observations were often extraordinarily close and consistent, certain aspects of photoperiodic induction, such as the fall in diapause in ultra-short photophases, were inexplicable in terms of the original form of the model. In particular, some of these observations seem to require that the clock is composed of many oscillations, or of damping oscillations (section C). Whilst this does not necessarily mean that the concept of a light-sensitive or photoinducible phase is invalid, it does raise the possibility that such a phase does not exist and that inductive control at the critical day length depends on some internal phase distortions within the circadian system. Since ultimate and unequivocal discrimination between the broad 'external' and 'internal' alternatives is still lacking we will proceed with the analyses of diapause induction in *S. argyrostoma* in terms of the external coincidence model, and consider the difficulties in Section F.

a) Similarities between eclosion and diapause induction: the 'hands' of the clock

The rhythm of pupal eclosion and the photoperiodic response in *S. argyrostoma* are alike in several respects: both are circadian and both show the same 'sensitive' or response periods for their initiation, entrainment, or induction (Saunders, 1979b, 1980b). They also show a close similarity, crucial to the external coincidence model, in their phase relationships to the light cycle. Figure 13.15, for example, shows the results of a 'wedge' experiment, exposing a series of cultures to a final photoperiod of 4 to 48 hours before their release into

DD. It then compares the phase relationship of the peaks of pupal eclosion with the peaks of high diapause observed in resonance experiments (see Fig. 11.10). In both cases the peaks occurred a fixed number of hours (+ multiples of 24 hours) after the end of the light period, once the latter exceeded about 12 hours. In other words, like a similar experiment with *Drosophila pseudoobscura* (Pittendrigh, 1966; Fig. 3.35), the circadian oscillators governing eclosion and photoperiodic induction are reset to a unique phase (equivalent to Ct 12) at the end of a protracted (> 12 hours) light pulse, and then free-run in darkness. This behaviour is essentially that described in the external coincidence model (Section A.3). Data from other sources indicate that ϕ_r (eclosion) occurs about 1 to 1½ hours after the photoinducible phase ϕ_i , and the strong similarities between eclosion and photoperiodic induction suggest that the former may be used in *S. argyrostoma* as overt 'hands' of the clock. The subsequent success in following this procedure amply bears this out.

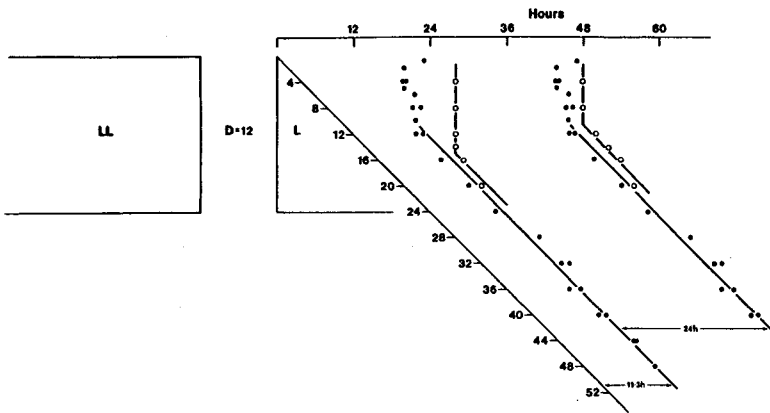


Fig. 13.15. *Sarcophaga argyrostoma*, phase relationship between peaks of pupal eclosion (closed circles) and peaks of high diapause from resonance experiments (open circles). For the determination of eclosion peaks, cultures pass horizontally, left to right, out of LL into 12 hours of darkness and then a final light phase of between 1 and 48 hours before release into DD. Peaks of high diapause are taken from Fig. 11.10. With both eclosion and diapause the oscillators take their principal time cue from the light-off ('dusk') transition once the final photoperiod exceeds about 12 hours. (From Saunders, 1976.)

(b) Use of eclosion phase-response curves for calculating photoperiodic phase relations

Phase response curves for single pulses of white light ($240 \mu\text{W cm}^{-2}$) of between 1 and 20 hours duration were obtained by exposing larval cultures of *S. argyrostoma* to the light-pulses at all phases of the free-running oscillation. Steady-state shifts in the phases of the eclosion peaks (Saunders, 1978a) were then observed. These data were then used to calculate theoretical steady-state phases of eclosion peaks (ϕ_r at Ct 23.0) and of ϕ_i (at Ct 21.5) in a variety of light regimes, using a computer program designed to accommodate all light pulses (1 to 20 hours), up to 4 pulses per 'cycle' (of any duration), with the first pulse in the train starting at all circadian times (Ct 01 to Ct 24), for up to nineteen consecutive cycles. The procedure adopted was essentially that of Pittendrigh's (1965) non-parametric model for *Drosophila pseudoobscura*, as outlined in Chapter 3. Particular attention was paid to the initial conditions (i.e. the starting phases) and to the rate at which entrainment to trains of pulses was achieved; both have proved to be of importance to the photoperiodic clock.

(c) *Parallels between entrainment and diapause induction in 'complete' and 'skeleton' photoperiods ($T = 24$ hours)*

Phase response curves for all pulse durations were used to compute the phase relationship of ϕ_i (Ct 21.5) to complete photoperiods (Fig. 13.16). The results showed that these computed phases were roughly parallel to 'dusk', consequently ϕ_i comes to lie in the dark with short photoperiods, but in the light with longer photoperiods (Saunders, 1978a). The point at which ϕ_i was calculated to pass the dawn 'threshold' corresponded very closely to the experimentally determined critical night length (LD 15½:9½). The similarity between this result and the external coincidence model is abundantly clear.

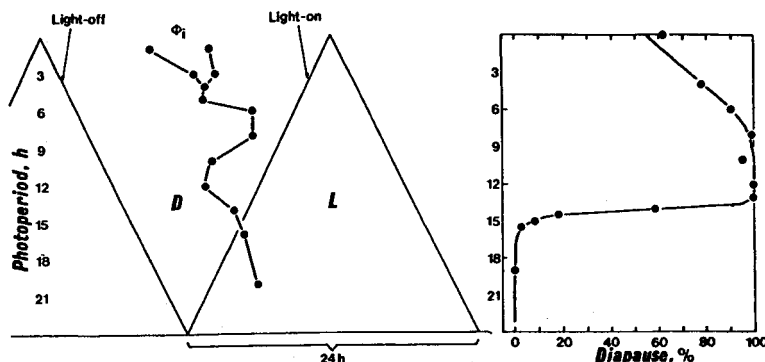


Fig. 13.16. *Sarcophaga argyrostoma*. The phase relationship of the putative photoinducible phase (ϕ_i) to complete photoperiods (on the left), computed from eclosion rhythm phase-response curves (see text), and compared with diapause induction data (on the right). When ϕ_i is computed to fall in the dark, diapause incidence is high; when ϕ_i crosses the dawn threshold, diapause is abolished. Note the similarity to the external coincidence model (Fig. 13.1). (From Saunders, 1981a.)

'Skeleton' photoperiods, consisting of two short pulses of light per cycle, are known to mimic many of the entraining effects (Chapter 3) and diapause-inducing effects (Chapter 10) of 'complete' photoperiods. Both symmetrical and asymmetrical skeletons have therefore been examined in *S. argyrostoma*, with comparisons between the positions of ϕ_i and diapause incidence made as before.

For symmetrical skeleton regimes, two 1-hour pulses of light were used (Fig. 13.17). As described in Chapter 3, C, each skeleton is open to two distinct 'interpretations' depending on which of the pulses is taken as the initiator and which the terminator of the simulated photoperiod. For example, a skeleton of PP_s10 (LD 1:8:1:14) can also be regarded as PP_s16 (LD 1:14:1:8). If the interval between the two pulses is small (or very large) the oscillator achieves a single, unique, phase relationship to the pulses in which the shorter interval is taken as the simulated photoperiod (= 'day'). In the ambiguous skeletons in the 'zone of bistability' (Pittendrigh, 1966; Chapter 3, C), however, the oscillation may take either of the intervals as 'day' depending on the phase of the oscillation which experiences the first pulse in the train. Figure 13.17 shows the results of entrainment to the full range of symmetrical skeletons, and Fig. 13.18 to the skeletons PP_s11 (LD 1:9:1:13) and PP_s15 (LD 1:13:1:9) in the 'zone of bistability'. In the latter case, adoption of PP_s11 results in long-night effects (high diapause)

whereas adoption of $PP_s 15$ results in short-night effects (low diapause). Both figures show that when ϕ_i is calculated to fall in the dark diapause incidence is high, whereas when ϕ_i falls in the light (or very close to a light-pulse) it is low. Once again there is no doubt that the observations are in accord with external coincidence.

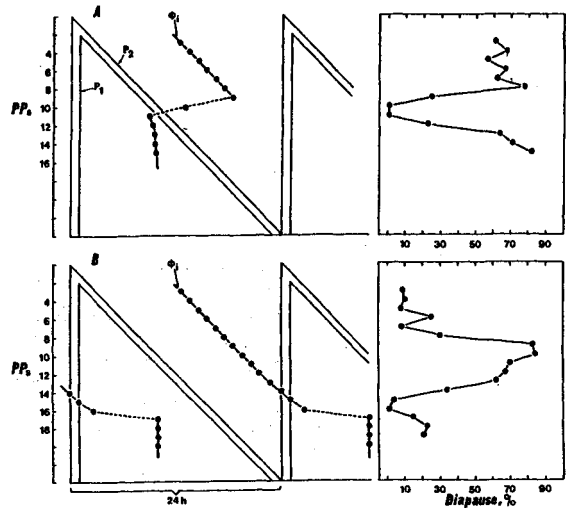


Fig. 13.17. *Sarcophaga argyrostoma*. Computed phase relationships of the photoinducible phase (ϕ_i) in symmetrical skeleton photoperiods (PP_s) comprising two 1-hour pulses of white light per cycle, compared with diapause incidence (on the right). A - first pulse (P_1) in the train starting at Ct 12; B - first pulse (P_1) starting at Ct 24 (or 0). A low incidence of diapause is observed when ϕ_i coincides with pulse 1 or pulse 2. (From Saunders, 1981a.)

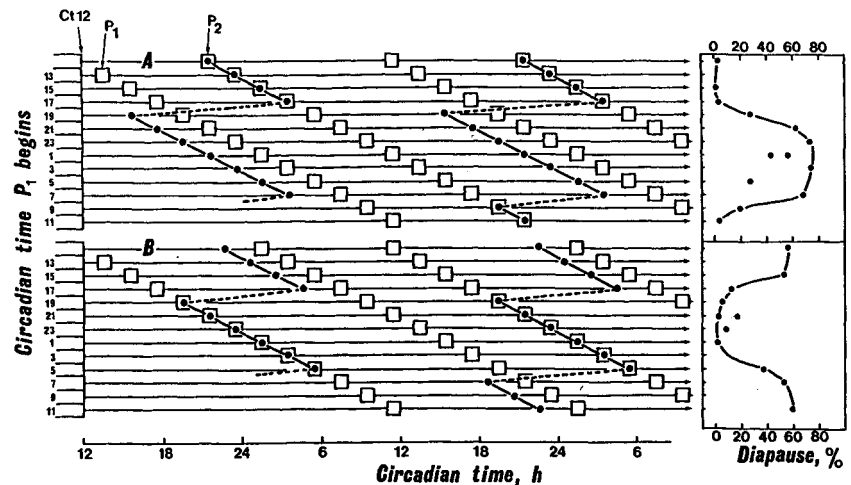


Fig. 13.18. *Sarcophaga argyrostoma*. Computed phase relationships of ϕ_i in skeleton photoperiods of $PP_s 11$ (LD 1:9:1:13) and $PP_s 15$ (LD 1:13:1:9) within the 'zone of bistability', with the first pulse in the train (P_1) starting at all circadian times (Ct 13 to Ct 11) following a transfer of cultures from LL to DD (\equiv Ct 12). At right, the incidence of pupal diapause (see also Fig. 11.15). Diapause incidence is high when ϕ_i falls in the dark, but is low when ϕ_i coincides with one of the two pulses making up the skeleton. (From Saunders, 1981a)

A number of asymmetrical skeletons have been analysed in this way: Fig. 13.19 presents the results for one of them (LD 10:14) with the 'night' systematically scanned by a second 1-hour pulse. The left-hand panel shows that ϕ_i (at Ct 21.5) is first phase delayed (to ψ_x), then phase advanced (to ψ_y) as the 1-hour pulse scans the night. In the middle of the night a phase discontinuity occurs with a phase-jump from ψ_x to ψ_y . (The dotted lines show the positions of the earliest and latest phase-jumps, respectively, depending on the phase (Ct) of the first pulse in the train: the solid line shows the position of the phase-jump for this particular experimental population). The right-hand panel shows the close correlation between the phase relationship of ϕ_i and diapause. When ϕ_i is delayed into the beginning of the main 10-hour photoperiod, and again when it coincides directly with the scanning pulse, diapause incidence is low; in all other places where ϕ_i falls in the dark it is high. These two low diapause positions correspond to the two points of 'sensitivity' (A and B) noted in night interruption experiments (Chapter 11), and the point of apparent 'insensitivity' between them represents the position of the maximum phase-jump. Interpretation in terms of entrainment of the circadian system is again consistent with the external coincidence model.

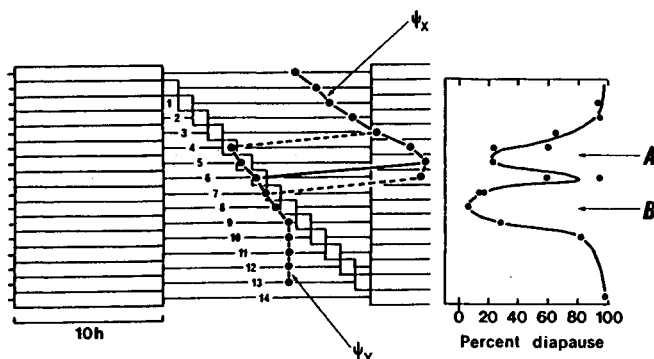


Fig. 13.19. *Sarcophaga argyrostoma*. Phase relationships (ψ) of ϕ_i in asymmetrical skeleton photoperiods (night-interruption experiments), composed of a 10-hour 'main' photoperiod and a 1-hour supplementary pulse scanning the 14-hour night, computed from eclosion PRCs and compared with diapause incidence (on the right). ψ_x and ψ_y are the two possible phase relationships (see text), and the dotted lines between them the 'earliest' and 'latest' phase jumps. The solid connecting line shows the phase-jump for the experimental population whose diapause responses are shown to the right. A and B - two points of low diapause characteristic of insect night interruption experiments (see also Fig. 13.21). See text for further details. (From Saunders, 1981a.)

(d) Entrainment and induction in cycles where T is not equal to 24 hours: the 'T-experiment'

In Chapter 3.C it was shown that when a circadian oscillator (period τ) becomes entrained to a light-cycle (period T) consisting of a single short pulse of light per cycle, the pulse must come to lie on that part of the phase-response curve which generates a phase shift equal to the difference between T and τ (Pittendrigh, 1965). It was this experimental observation which led to the so-called 'T-experiment' (Pittendrigh and Minis, 1964, 1971; Section E. I (b)), the rationale of which was that when T was less than τ the pulse would fall in the late subjective night, whereas when T was greater than τ the pulse would fall in the early

subjective night. Consequently, an alteration in T would cause the light-pulse to illuminate different phases of the oscillation, in the absence of any other photoperiodic influence. This experiment was one of those producing equivocal results with *Pectinophora gossypiella* (Section E. 1 (b)); here we will examine similar, but in this case successful, experiments with *S. argyrostoma*.

Cultures of larvae derived from female flies maintained in long nights, and therefore 'preconditioned' for pupal diapause, were set up in a series of light-cycles from $T = 21.5$ to $T = 30.5$ hours, all containing a single 1-hour pulse of light. The phase response curve for 1-hour pulses was used to compute where the first pulse in the train must fall, and which T -value must be used, to illuminate each hour of the subjective night. The results (see Fig. 11.13) showed that only in a cycle of $T = 21.5$ hours (LD 1:20.5) in which the light-pulse came on at Ct 20 and finished at Ct 23.5 - and therefore illuminated ϕ_i (at Ct 21.5) - were short-night, or low diapause effects obtained. In all other regimes ϕ_i fell in the dark and diapause was high (Saunders, 1979a).

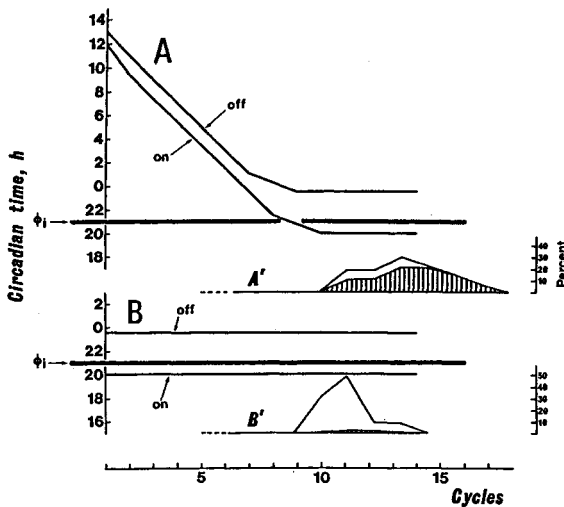


Fig. 13.20. Rate of approach to steady-state entrainment in two populations of *Sarcophaga argyrostoma* larvae exposed to cycles of LD 1:20.5 (T 21.5 hours). A - first pulse in the train starting at Ct 12, B - first pulse starting at Ct 20. A' - distribution of puparium formations in population A; B' - ditto for population B; shaded portions of polygons represent diapause pupae. In B, the photoperiodic oscillation achieves immediate steady state with the 1-hour pulse coming on at Ct 20 and leaving the oscillation at Ct 23.5. ϕ_i (at Ct 21.5) is therefore illuminated in each cycle, larval development is rapid, and diapause incidence low. In A, the oscillation passes through about nine non-steady-state or transient cycles before reaching steady state and coinciding with ϕ_i . Consequently, larval development is protracted and diapause incidence high. (From Saunders, 1979a.)

Since the phase-response curve for 1-hour pulses in *S. argyrostoma* is of Winfree's (1970) weak Type 1, the circadian pacemaker may pass through many transient cycles before reaching steady state (Saunders, 1978a). The number of such transients depends on the phase (circadian time) at which the first pulse in the train occurred. Attention to *initial* conditions, therefore, provides us with another kind of T -experiment. For example, all cultures exposed to $T = 21.5$ hours (LD 1:20.5) eventually achieve a steady state in which the light-pulse illuminates Ct 20

to 23.5. However, those cultures starting at Ct 20 do so immediately, and those cultures starting at Ct 12 pass through nine transients before the light pulse coincides with ϕ_i . Cultures starting at other phases require an intermediate number of transients. Since photoperiodic induction in *S. argyrostoma* (and most other insects) involves a summation of photoperiods (see Chapter 12), and the early larval instars are more 'sensitive' to photoperiod than older larvae (Saunders, 1980b), one may predict a positive correlation between the number of transients and diapause incidence. Results (Fig. 13.20) show this to be the case, with cultures starting at Ct 20 producing 3.0 and 0.4 per cent diapause, and those starting at Ct 12 producing 66.0 and 84.7 per cent.

Since the photoperiodic oscillation is being probed by a single short pulse of light, in the absence of any other photoperiodic influence, these results offer strong circumstantial evidence for the 'reality' of the photoinducible phase - although they do not provide an unequivocal distinction between external and internal coincidence.

(e) Asymmetrical skeletons or night interruptions when T is not equal to τ

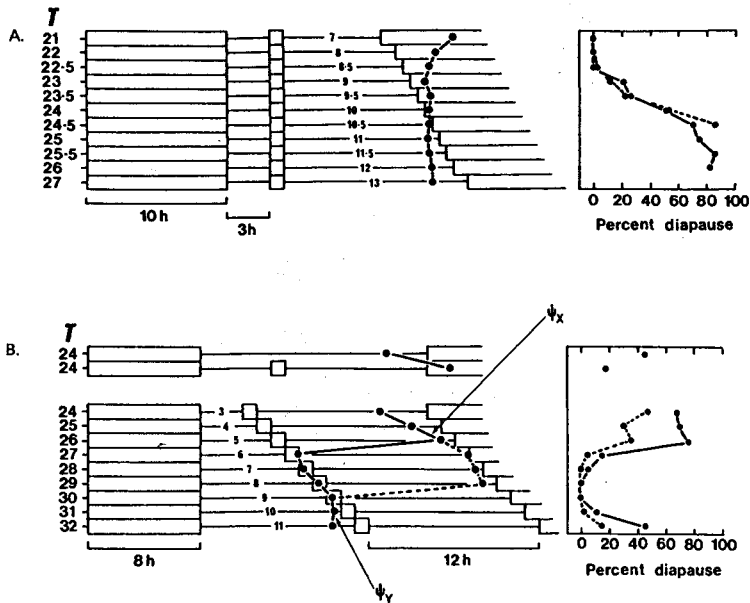


Fig. 13.21. *Sarcophaga argyrostoma*. Computed phase relationships of ϕ_i and diapause incidence in asymmetric skeleton photoperiods whose periods (T) are not equal to 24 hours. Upper panels: the supplementary pulses (1 hour) are placed 3 hours into the night (at a point equivalent to A in Fig. 13.19); the hours of darkness following this pulse are then varied from 7 to 13 hours. Lower panels: the supplementary pulse scans the night but is followed in all cases by a terminal dark period longer than the critical night length (see Fig. 11.17 for similar designs in *Megoura*). ψ_x and ψ_y are the two possible phase relationships, the connecting lines (solid and dotted) the 'earliest' and 'latest' phase jumps between them. Right hand panels show that diapause is high when ϕ_i falls in the dark but is low when illuminated. (From Saunders, 1981a.)

Two experiments have been conducted using the night interruption technique in non-24-hour cycles (Saunders, 1979a). These experiments were based on designs used by Lees (1970, 1971b) for *Megoura viciae* but, in *S. argyrostoma*, and within a framework of external coincidence, provide strong experimental evidence that ϕ_i (if a real entity) is at 'point B' rather than 'point A' in the characteristically bimodal response. The first of these experiments used a series of asymmetrical skeletons in which the supplementary pulse was placed 3 hours after the end of the 'main' 10-hour photophase, at a point equivalent to 'A', and the terminal hours of darkness were then systematically varied. In the second, using an 8-hour 'main' component, the hours of darkness before the supplementary pulse were varied, but the hours following it were maintained at 12 hours, or longer than the critical night length. Phase-response curves for the light-pulses involved in these experiments were used to compute the steady-state phase relationships of ϕ_i to the light regimes. The designs of these experiments may be seen in Fig. 13.21.

In the first experiment (Fig. 13.21A) regimes in which the terminal hours of darkness were less than about 9 hours gave a low incidence of diapause, but as the terminal dark hours rose the incidence of diapause followed. In the second type of experiment (Fig. 13.21B) diapause incidence was high until the supplementary pulse fell about 6 to 9 hours after dusk, after which it rose again. In Lees's (1971) interpretation (Chapter 11,C), the first experiment produced a short-night response until the terminal hours of darkness exceeded the critical night length, whereas the second experiment produced a long-night response until the pulse fell in a position of the night equivalent to point B. The photobiological effects of light falling on A were therefore *reversible* by a dark period longer than the critical, but those at B were not.

Such an interpretation is also valid for *S. argyrostoma*, but here (Fig. 13.21) the results may be interpreted by whether ϕ_i is illuminated or not – or in terms of external coincidence. Thus in Fig. 13.21A, ϕ_i falls in the light until the terminal hours of darkness exceed about 10.5 hours; and in Fig. 13.21B, ϕ_i falls in the dark until the oscillation phase-jumps (in LD 8:6:1:12) so that ϕ_i coincides with the 1-hour pulse. Once again computed positions of ϕ_i in relation to the light were in very close agreement with diapause incidence. The results also provide strong evidence that ϕ_i is indeed at B (and not A), as proposed by the external coincidence model (Pittendrigh, 1966).

3. Overt rhythms as 'hands of the clock'

The foregoing sections show that phase response curves for the pupal eclosion rhythm of *S. argyrostoma* may be used to predict the phase relationship of the supposed photoinducible phase (ϕ_i) to the light cycle in studies of diapause regulation. In every case, prediction that ϕ_i fell in the light led to a low diapause incidence, whereas prediction that ϕ_i fell in the dark led to high diapause. There is no doubt that application of this technique – to *S. argyrostoma* – was met by considerable success, lending strong support to the hypotheses that (a) photoperiodic time measurement was a function of the circadian system, and (b) that a form of external coincidence is entirely appropriate in the *Sarcophaga* case.

In hindsight, however, the success of these experiments may have been due to the periods (τ values) and phase response curves (PRCs) of the eclosion and photoperiodic oscillators being similar. For the latter, 1-hour pulse PRCs were both of the low 'amplitude' Type 1 (see Chapter 11, B. 3). In some other species, overt rhythmicity has been less useful as a measure of phase. These cases will be reviewed here.

In the pink boll worm moth, *Pectinophora gossypiella*, clear differences between overt rhythmicity (pupal eclosion, egg hatch and oviposition) and photoperiodic induction were

noted (Pittendrigh and Minis, 1971). This may be attributed to the different overt rhythms and diapause regulation being regulated by different pacemakers, with different properties, within the multioscillator circadian system.

Similar differences were observed with the blow fly *Calliphora vicina* (Kenny and Saunders, 1991; Saunders, 1998). Working with the adult locomotor activity rhythm and photoperiodic induction of larval diapause, it was shown that, although both systems were operating simultaneously in the female fly, there were distinct differences between them. Locomotor rhythmicity displayed a free-running periodicity (τ) of about 22.5 hours and was fully self-sustained, persisting for the life of the fly. The photoperiodic oscillator(s), on the other hand - as revealed by Nanda-Hamner experiments - showed inter-peak intervals (τ) much closer to 24 hours, and bore all the hallmarks of a damping system. It was concluded that although locomotor rhythmicity could be used in a very general way as 'hands' of the photoperiodic clock, the two were probably 'separate' parts of the circadian system with quite different properties. Lankinen (1986a, b) and Lankinen and Riihimaa (1992) came to a similar conclusion with pupal eclosion and photoperiodism in *Drosophila littoralis* and *Chymomyza costata*.

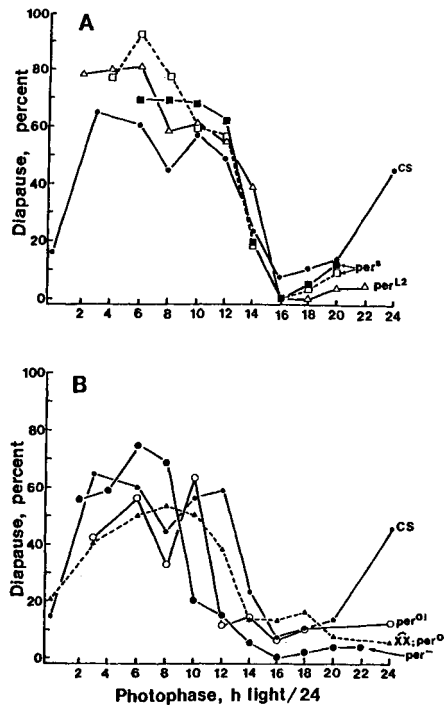


Fig. 13.22. *Drosophila melanogaster*, ovarian diapause showing photoperiodic response curves. A – wild type flies (Canton-S), short period mutant (*per^S*) and long period mutant (*per^{L2}*). B – wild type (Canton-S), behaviourally arrhythmic mutant (*per⁰¹*), flies deleted of the entire *period* gene (*per⁻*), and 'attached-X' *per⁰¹* flies. Note that the critical day length is the same in wild type flies and both short and long period mutants (A), and that arrhythmic flies also distinguish short from long days (B), although with a shorter critical day length. (From Saunders, 1990).

More direct evidence for the 'separate' nature of overt rhythmicity and photoperiodic induction came from a study of the *period* mutants of *Drosophila melanogaster*. In this species it was found that critical day lengths for ovarian diapause induction in *period* mutants (*per^S* and *per^{L2}*) were the same as that for Canton-S wild type (Saunders et al., 1989; Saunders, 1990). Furthermore, behaviourally arrhythmic flies carrying *per^O*, or those devoid of the *per* gene (*per⁻*), were apparently still capable of distinguishing short days from long days, albeit with a shortened CDL (Fig. 13.22). It was concluded that the *per* gene and its product, although part of the feedback loop governing overt circadian rhythmicity (Chapter 4), had little to do with photoperiodic timing, the latter being regulated by a 'separate' part of the insect's multi-oscillator circadian system. The idea that circadian feedback loops exist that do not incorporate the *period* gene gains credence with the recent observation that the 'morning' (M) peak in the locomotor activity rhythm of *D. melanogaster* is also *per*-independent (Helfrich-Förster, 2001).

In summary, notwithstanding the success of the technique with the flesh fly *Sarcophaga argyrostoma*, the use of overt circadian rhythmicity as a measure of phase within the covert photoperiodic system offers only broad parallels and should therefore be approached with caution.

E. WHAT IS THE EVIDENCE FOR A SPECIFIC PHOTOINDUCIBLE PHASE?

Given the apparent success of analyses of photoperiodic induction in *S. argyrostoma*, in terms of the external coincidence model (see Section C; Saunders, 1978a, 1979a), it is tempting to conclude that such a model accounts for the phenomenon, and that the photoinducible phase (ϕ_i) is a physiological 'reality'. However, there is still no *unequivocal* way to distinguish between 'external' and 'internal coincidence', and the data for *S. argyrostoma*, and other species, could theoretically be explained by either model. The existence of ϕ_i has also been called into question by several authors.

Night interruption experiments in which the scotophase of a diapause inducing cycle is systematically scanned by a short supplementary light pulse (see Chapter 11, section B1), frequently reveal two phases of sensitivity or 'short night' effect, called A and B (Fig. 13.19). Point B is often larger than A, as in *S. argyrostoma* (Saunders, 1979a). In terms of the external coincidence model (see above), a light pulse falling on A is thought to cause a phase delay of the circadian pacemaker, driving the photoinducible phase (ϕ_i) from the late subjective night into the main photophase. Coincidence with dawn light then leads to a low incidence of diapause. Light falling at B, on the other hand, causes phase *advances* and a second point of low diapause (B) by direct coincidence of light and ϕ_i . The photoinducible phase, therefore, is thought to lie at B rather than at A.

However, in some species, such as the cabbage butterflies *Pieris brassicae* (Goryshin and Tyshchenko, 1968) and *P. rapae* (Barker et al., 1964), and the codling moth *Carpocapsa pomonella* (Peterson and Hamner, 1968), point B was found to be *smaller* than A. Point B may also be smaller than A in the flesh fly *Sarcophaga crassipalpis* (Gnagey and Denlinger, 1984), and negligible or even absent, as in the swallowtail butterfly *Papilio xuthus* (Hidaka and Hirai, 1970) and the ground beetle *Pterostichus nigrity* (Thiele, 1977a).

Working with *P. brassicae*, Brunnarius and Dumortier (1984, 1987) also confirmed that point B was less prominent than A, and that B disappeared entirely under some photoperiods, at temperatures below about 19°C, or if the larvae were raised on a semi-synthetic diet rather than cabbage. These results led Dumortier and Brunnarius to question the existence of ϕ_i (at point B), and therefore the veracity of the external coincidence model. They rejected the idea

that ϕ_i could be at A however, because the short night effect of light falling on A was reversed by increasing the terminal hours of darkness, as in *S. argyrostoma* (Saunders, 1979a). Point A in *P. brassicae* also bore no fixed phase relationship to either dawn or dusk.

The important question whether ϕ_i is at A or B – or, indeed, whether the photoinducible phase is a physiological ‘reality’ – may be resolved by supposing differential light sensitivities for the two postulated effects of light: phase resetting and photoinduction (the diapause switch). If phase shifting occurs at lower light intensity than photoinduction, point A (phase delay) would persist at intensities where point B (phase advance and photoinduction) would become smaller, eventually to disappear. Sensitivity to light for photoinduction might also be less at lower temperature, or on certain larval diets, thereby explaining some of the *P. brassicae* data. Yoshida and Kimura (1993), in considering similar problems with *Drosophila triauraria*, also concluded that variations in the size of A and B may be due to variations in the length and photosensitivity of the A and B phases. It is concluded here that ϕ_i – and the external coincidence model – remain valid concepts in insect photoperiodic time measurement.

Lavialle and Dumortier (1990) produced physiological evidence possibly consistent with the concept of a specific photoinducible phase. Using cytochrome oxidase activity as a marker of energy metabolism within the pupal brain of *P. brassicae*, they concluded that the level of metabolism in the optic centres was connected with the photoperiodic response, and that points A and B could be discriminated at a functional level. Larvae maintained at 21°C and fed on cabbage leaves were exposed to LD 13:11 with supplementary 1 hour light pulses early in the night (at A), late in the night (at B), or at a ‘neutral’ position in the middle (X); non-pulsed larvae served as additional controls. Most intense staining was observed in the optic centres when larvae were pulsed at either A or B. The level of staining was less at X, and the non-pulsed controls showed lower staining at B and none at A. It was concluded that coincidence of light with point B resulted in an increase in cytochrome oxidase activity. Whilst these results do not distinguish unequivocally between ‘internal’ and ‘external’ coincidence, they are certainly consistent with the latter and these experiments deserve repetition and development.

F. CURRENT STATUS OF THE MODELS

Photoperiodic clock models (Table 13.1) have been developed over the years to incorporate accumulating experimental data into a sound experimental (largely circadian) framework. The latter includes the principles of entrainment to light and temperature cycles, the multioscillatory nature of the circadian system, and other ‘canonical’ properties such as persistence in constant conditions and the temperature compensation of circadian period. What has emerged is a clear indication that aspects of photoperiodic time measurement (PPTM) may be important functions of the circadian system: Bünning’s *general* hypothesis is therefore largely valid. Hourglass-like timers, formerly thought to represent a completely different kind of ‘clock’ probably represent rather heavily damped circadian oscillators, otherwise presenting features in common with clearly circadian-based devices. Even ‘classical’ hourglass timers, such as that thought to govern seasonal morph determination in the vetch aphid *Megoura viciae*, have turned out to present some circadian properties (Vaz Nunes and Hardie, 1993).

When it comes to more explicit versions of Bünning’s general hypothesis the interpretation of experimental data in terms of clock models becomes less certain. Although there is persuasive – but still *circumstantial* – evidence suggesting some sort of external coincidence in some species (e.g. *Sarcophaga argyrostoma*) and internal coincidence in others, there is

currently no unequivocal way to distinguish between these types of model. The hourglass clock-oscillator counter model (Vaz Nunes and Veerman, 1982) also provides a persuasive possibility for many photoperiodic phenomena, not only in the spider mite. Other propositions such as the ‘amplitude model’ (Pittendrigh et al., 1991) remain to be tested experimentally, rather than by just computer simulation. The most recent model - the ‘double circadian oscillator model’ (Vaz Nunes, 1998) – rests on a sound experimental basis and has been extensively tested by simulation. In its details, however, this model (see Section A, 4) differs fundamentally from external and internal coincidence. There is, therefore, a variety of plausible models available, all of which present attractive features. No doubt shortcomings will be found with all of them when new experimental protocols are devised to test them. These shortcomings will result in further development of hypotheses and models, as has happened before, and will bring us another step forward in our search for the mechanism of photoperiodic time measurement.

In summary, we still do not understand how the circadian system plays its part in PPTM. It is becoming clear, however, that night length measurement by the photoperiodic clock and overt behavioural rhythmicity (erstwhile ‘hands of the clock’) are quite ‘separate’ systems (section D, 3), with the former possibly even being distinct from the *per-tim* molecular loop described in Chapter 4. The rhythmic outcomes of Nanda-Hamner and Bünsow experiments (Chapter 11) may also reflect a part of the circadian system that is ‘separate’ from night length measurement since, in several species, there is only very weak correlation between them especially over a south-north cline (Veerman, 2001). Thirdly, it is also possible that Nanda-Hamner interpeak intervals are ‘separate’ from those parts of the circadian system regulating overt behavioural rhythms. All of these possibilities and complications, however, *do not necessarily mean* that PPTM is carried out by an hourglass device, separate from the circadian system. An alternative hypothesis - presented in this book - is that night length measurement is a function of a damping circadian oscillation, in some species so heavily damped that it presents the *properties* of an hourglass-like timer.

A single, conserved, circadian-based, model *could* account for the phenomenon of PPTM in all species. Much more likely, however, is a common circadian basis (Bünning’s general hypothesis) with considerable evolutionary divergence from this origin. We might therefore expect diversity in detail, with the principles of external coincidence, internal coincidence, and hourglass-like responses preserved in different insect species. We will return to some of these problems of photoperiodic time measurement in Chapter 16.

ANNOTATED SUMMARY

1. Models for photoperiodic time measurement (PPTM) are outlined (Table 13.1). These include hourglass-like timers and those based on the circadian system. The latter include various propositions in which circadian oscillations have either a ‘non-clock’ role operating at a downstream (e.g. ‘counter’) level, or a ‘clock role’ in which they are directly involved in night length measurement. The most prominent of the latter are ‘external coincidence’, ‘internal coincidence’, and the most recent ‘double circadian oscillator’ model.
2. The most successful models are those that are firmly based on experimental evidence and sound circadian principles. The best models incorporate entrainment theory, ‘canonical’ circadian properties such as persistence (or damping) under constant conditions, temperature compensation of period (τ), and the accumulation of successive cycles by the photoperiodic ‘counter’ (see Chapter 12). Also essential is extensive computer simulation

and further experimental testing which separate effective models from less well founded propositions.

3. Some kind of multi-component 'internal coincidence' device is, *a priori*, an attractive model for the photoperiodic clock. Circumstantial evidence for such a clock has been obtained in the parasitic wasp *Nasonia vitripennis* which may have separate 'dawn' and 'dusk' oscillators, and in which light seems to play no direct or essential role in induction. Consequently, diapause- and development-promotion can occur in the complete absence of light, or after transfer from LD to DD.
4. The 'external coincidence' model, however, adequately explains photoperiodic time measurement in the flesh fly *Sarcophaga argyrostoma*. In this model, the oscillatory component (probably composed of several circadian oscillators) behaves in almost exactly the same way as that governing pupal eclosion in *S. argyrostoma* and *Drosophila pseudoobscura* (see Chapter 3). Thus, it is 'damped out' by photophases in excess of about 10 to 12 hours, but restarts at dusk to measure night length as if it were an 'hourglass'. Only in greatly extended nights does the system reveal endogenous circadian properties (see Chapter 11). In natural 24-hour cycles containing a 'day' longer than about 12 hours, diapause is promoted when the accompanying dark period is greater than the critical night length (9½ hours), but diapause is eliminated when the dawn transition coincides with a particular light-sensitive phase (ϕ_i) occurring 9½ hours after dusk. In many other insects night length also occupies a similar 'central' role in photoperiodism.
5. The external coincidence model in its original form (Pittendrigh, 1966), however, fails to explain certain features of the photoperiodic response in *Sarcophaga argyrostoma*, particularly the fall in diapause incidence in ultra-short (< 8 hours) photoperiods, and the low incidence of diapause in cultures transferred from LL into DD. In both types of regime, ϕ_i should fall in the dark in each circadian cycle, and diapause incidence should remain high. Some of these discrepancies, however, are explained by the presumed multi-oscillator 'construction' of the *Sarcophaga* clock, with the efficiency of night length measurement being a function of the internal temporal synchrony between the various components. Alternatively the photoperiodic clock in *Sarcophaga* might comprise damping oscillators.
6. The use of overt behavioural rhythms as 'hands' of the photoperiodic clock is reviewed. This procedure has been most successful in *S. argyrostoma* where phase response curves for the pupal eclosion rhythm have been used to predict phase relationships between the postulated photoinducible phase (ϕ_i) and light in a wide range of simple and complex light cycles. These data offer strong, although still circumstantial, evidence for (a) the circadian nature of PPTM in this species, and (b) for some sort of 'external coincidence'. In other species, however, results indicate that overt rhythmicity and PPTM are governed by *separate* components of the multi-oscillator circadian system, and that similarities between them may only be used as a broad comparison.
7. The separate nature of overt rhythm control and PPTM has been demonstrated in *Drosophila melanogaster* by a study of ovarian diapause induction in *period* mutant flies. Behaviourally arrhythmic flies (either *per*^O or those lacking the entire *period* gene) were found to retain the ability to discriminate between short and long days, indicating that the *period* gene was not causally involved in PPTM.
8. Evidence for a specific photoinducible phase (ϕ_i) is reviewed. Problems with the interpretation of 'night interruption' experiments, particularly the 'disappearance' of 'point B' that, in external coincidence, is taken to mark the phase of ϕ_i , is explained by probable differences between photic sensitivities of phase shifting and photoinduction. Experiments using cytochrome oxidase activity as a marker of energy metabolism in the brain may

provide an experimental procedure to move these observations from a phenomenological to a physiological level.

9. The most recent clock-counter model is based on observations that the measurement of 'long' and 'short' nights may be fundamentally different, particularly concerning temperature effects on the counter mechanism. The 'double circadian oscillator' model proposes two oscillators, each determining the length of the night, but one measuring it as 'long' and the other as 'short'.
10. Time measurement in insect photoperiodism is clearly diverse: it is possible that a continuous 'spectrum' of clocks has evolved. All may be based on circadian rhythmicity so that Bünning's general hypothesis is broadly correct. Most show some evidence of structural complexity, and at least two components are theoretically necessary to account for all aspects of the phenomenon. At one end of the spectrum lies *Nasonia vitripennis* with putative dawn and dusk oscillators. In the middle are species like *Sarcophaga argyrostoma* in which a (presumably multioscillator) circadian component appears to measure night length from dusk as if it were an hourglass, but shows circadian properties in extended periods of darkness. At the other end of the spectrum are insects like *Megoura viciae* in which obvious circadian properties may be lost, night length being measured by an apparent hourglass. The *Megoura* type may be derived from an oscillatory clock of the external coincidence type by a rapid 'damping' of the circadian oscillator and the loss of the redundant property of its free-running behaviour. The similarities and differences between the various photoperiodic clocks are discussed. All probably have their origins in the circadian system, but have undergone subsequent evolutionary divergence.

This Page Intentionally Left Blank

CHAPTER 14

PHOTOPERIODIC PHOTORECEPTORS AND CLOCK LOCATION

Four seasons fill the measure of the year/There are four seasons in the mind of man.

John Keats

CONTENTS

Introduction	433
A. <i>Light Input Pathways</i>	434
1. Extraoptic photoreception	434
(a) Elimination experiments	434
(b) Supplementary illumination	435
(c) Transplantation and <i>in vitro</i> illumination	438
2. The role of the compound eyes	441
3. Carotenoids and the role of vitamin A	442
4. Opsins and the phototransduction cascade	443
B. <i>Photoperiodic Clock Location and Output</i>	444
1. Neurobiological studies	444
2. Melatonin as a photoperiodic clock output?	446
Annotated Summary	447

INTRODUCTION

SIMPLE models for photoperiodic phenomena include a *photoreceptor* to distinguish light from dark, a *clock* to 'measure' the length of the day or night, and an *output* to regulate the seasonal response. Seasonally appropriate phenomena such as diapause and polyphenism, their endocrine regulation, the properties of the clocks that control them, and the relationship of these clocks to the circadian system are discussed in Chapters 9 to 13. Here we now address the problem of the anatomical locations of photoperiodic clocks and the photoreceptors linking them to the photic environment. The model briefly outlined above suggests a simple linear information flow through **photoreceptor→clock→output**, broadly similar to that for the regulation of overt circadian rhythms (see Chapter 8). That this model is a gross oversimplification will become apparent; the complexity of the system will be discussed further in Chapter 16.

Consideration of this simple model for *overt* rhythms (see Chapter 8) has led to advances in identifying the location of circadian photoreceptors and clocks. For example, it may be predicted that occlusion or removal of the photoreceptor would result in free-running,

but still rhythmic, behaviour, whereas removal of the clock would result in behavioural arrhythmicity. In photoperiodism, however, it is not so easy to distinguish these effects since elimination of the photoreceptors and elimination of the clock would *both* result in an inability of the insect to distinguish between long and short days. For this reason most of the experiments described in this chapter fail to make this distinction, and discussion of photoreceptor and clock location will, in many cases, be treated together.

In a recent review of arthropod photoperiodic receptors, Numata et al. (1997) considered three experimental procedures. These were: (1) *elimination*, in which candidate photoreceptors are removed by surgery or cautery, or are covered by an opaque material. (2) *supplementary illumination*, in which different parts of the insect body receive additional light often through a 'light guide'. And (3) *transplantation*, where candidate organs are transplanted from one part of the body to another, or are removed to *in vitro* conditions, and then illuminated. All three of these techniques will be described here.

A. LIGHT INPUT PATHWAYS

1. Extraoptic light reception

(a) Elimination experiments

Early data for photoperiodic clocks suggested that 'organised' photoreceptors (compound eyes and ocelli) were not involved in the response to light. Tanaka (1950), for instance, cauterised the lateral ocelli (stemmata) of fourth instar larvae of the silk moth *Antheraea pernyi*, causing the complete disappearance of these organs in the next instar. Nevertheless, photoperiodic sensitivity of these 'blinded' larvae was unimpaired, short-day exposure leading to the production of diapausing pupae. De Wilde et al. (1959) blinded newly emerged adults of the Colorado potato beetle, *Leptinotarsa decemlineata*, either by cautery or by covering the compound eyes with an opaque black paint, and similarly found no interference with the photoperiodic response. Comparable experiments were carried out with the vetch aphid *Megoura viciae* (Lees, 1964) and the grasshopper *Anacridium aegyptium* (Geldiay, 1971), with similar results. More recently, Shimizu (1982) showed that covering the larval stemmata of *Bombyx mori* also failed to interrupt the photoperiodic response. On the other hand, covering the entire larval head with black paint prevented the diapause averting action of LD 20:4 (1 lux, but not 10 lux) (Shimizu and Hasegawa, 1988). In this respect, therefore, photoperiodic clocks were considered to be similar to those controlling eclosion rhythms with regard to brain photoreception, but different from those controlling, say, the locomotor activity rhythm in cockroaches. The data were a strong indication that compound eyes and ocelli were not involved, suggesting extraoptic or extraretinal light sensitivity.

Larval diapause in the blow fly *Calliphora vicina* is induced by short day illumination of the adult female flies (Vinogradova and Zinovjeva, 1972b). Using microsurgery, Saunders and Cymborowski (1996) effectively isolated the compound eyes from the central brain of this species by complete bilateral removal of the optic lobes. Despite this operation, flies were found to differentiate short days from long days by producing diapause or nondiapause progeny (Fig. 14.1), demonstrating that the compound eyes were *not essential* for photoreception. Microcautery of the ocelli, or covering them with an opaque black wax, was also without effect. In *C. vicina*, therefore, the central brain emerged as a likely site both for the clock and its photoreceptors (but see Section B for the related blow fly, *Protophormia terraenovae*).

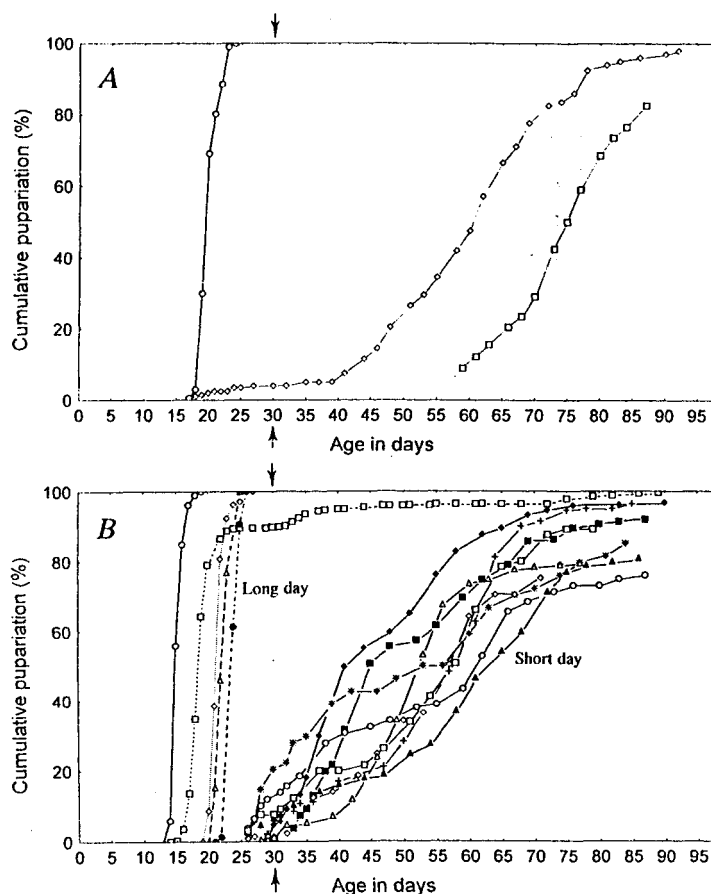


Fig. 14.1. *Calliphora vicina*. Cumulative pupariation curves for the larval progeny of (A) unoperated (control) female flies exposed to long day length (LD 18:6, 20°C; open circles) or short day length (LD 12:12, 20°C; open lozenges and squares); and (B) lobectomised females exposed to long day length (5 cultures, left hand group), short day length (8 cultures, right hand group) or to continuous darkness (closed squares). All larval cultures were raised in darkness at 11 to 13°C. Larvae failing to pupariate by day 30 post eclosion (arrow) were considered to be in diapause. (From Saunders and Cymborowski, 1996).

(b) Supplementary illumination

An alternative approach to the problem of the location of photoreceptors is to illuminate different parts of the body to determine the site of organs capable of differentiating long from short days. This has been done in at least three insects. Working with larvae of the pine lappet moth *Dendrolimus pini*, Geispits (1957) covered either the head or the abdomen with an opaque hood for 12 hours each day and then exposed the larvae to continuous illumination. Those larvae with the abdomen covered showed a long-day response, but those with the head

covered behaved as though they were in LD 12:12 and entered diapause. These experiments, therefore, showed that the photoperiodic receptors were located in the head.

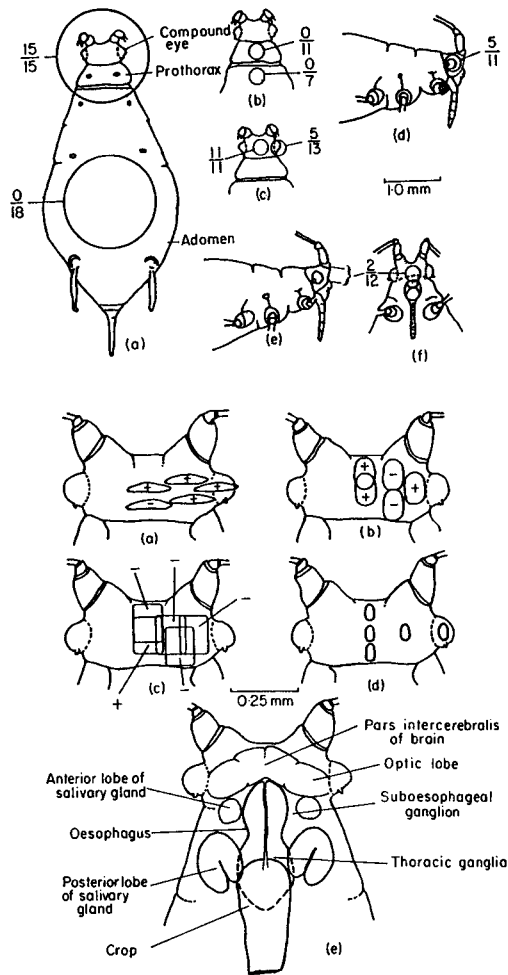


Fig. 14.2. The location of the photoperiodic receptors in *Megoura viciae*. The upper group of figures shows the effect of directing light at the aphids with capillary illuminators. The denominator in each 'fraction' indicates the number of aphids tested, the numerator the number responding positively to the supplementary illumination to give a long-day response. The lower group of figures shows the results obtained with light-conducting filaments. Positive and negative responses are indicated by plus and minus signs. (From Lees, 1964.)

A. D. Lees (1960b, 1964), working with the green vetch aphid, *Megoura viciae*, reached a similar conclusion. In some of the most elegant experiments in biology, Lees maintained parent aphids (virginoparae) in a short day regime (LD 14:10). He then supplied a 2-hour period of supplementary illumination to different areas of the head and body by means of micro-illuminators constructed from fine metal capillaries or plastic filaments (light guides)

drawn from a viscous solution of polystyrene in benzene. The aphids were attached to the micro-illuminators for 2 hours each day; the rest of the time they were allowed to feed undisturbed on the host plant. The rationale behind this approach was that if the micro-illuminator was placed in a position to stimulate the relevant photoreceptor the aphids would react to a long day length (LD 16:8) and produce virginoparous daughters. On the other hand, if the photoreceptors were not illuminated, the aphids would switch to the production of oviparae. The results obtained with capillaries (Fig. 14.2) showed that illumination of the head induced virginoparae, whereas illumination of the thorax or abdomen induced oviparae. This showed that the photoreceptors were in the head. Since the embryos within the abdomen failed to respond, the results also demonstrated that the controlling mechanism was strictly maternal. Smaller bore illuminators revealed that the most sensitive area of the head was in the midline of the dorsum. A smaller proportion of the aphids showed a long-day response when the lateral parts of the head or the compound eyes were illuminated. Beams of light directed at the front of the head, or directly into the compound eyes, were also less effective than a light beam directed vertically on the dorsal midline of the head capsule. Since no dorsal ocelli are present in viviparous aphids and the cuticle in this region is light amber and relatively transparent, Lees concluded that the photoperiodic receptors were probably in the underlying protocerebrum. Furthermore, he suggested that the neurosecretory cells of the *pars intercerebralis* might be implicated both as receptors and as humoral effectors. Illumination of the abdomen of parent aphids had no effect on the determination of progeny-type. Nevertheless, such illumination of the grandparents showed that the photoperiodic mechanisms of the (parent) embryos during the last 4 days of prenatal development were fully accessible to light transmitted through the body wall of the grandparent.

Further progress in the location of the photoperiodic mechanism in *M. viciae* was made by Steel and Lees (1977) using selective radio-frequency microcautery of the brain. Destruction of the five neurosecretory cells (NSC) making up group I on the dorsal aspect of the protocerebrum abolished responses to changed day length, whereas extensive damage to other groups of NSC, or to the compound eyes or optic lobes, was without effect. Group I NSC were therefore considered to be the photoperiodic *effectors*, secreting a 'virginopara-promoting' substance, possibly passing down the axons of these cells, through the suboesophageal ganglion and ventral nerves to the vicinity of the ovarioles. In addition, since areas of the brain slightly lateral to the Group I NSC were also required for the photoperiodic effects, these areas were thought to contain the neuronal clock regulating the release of the neurosecretory material.

Working with *Anacridium aegyptium*, a species showing an ovarian diapause of the long-day type, Geldiay (1971) exposed the central part of the head or the abdomen to 6 hours of supplementary illumination after a general body illumination of 9 hours. When the head was thus stimulated the nuclear diameters of the A and B neurosecretory cells, and the oöcytes, were larger than in those insects with the abdomen illuminated. She concluded that long-day illumination activated the neurosecretory cells having a positive control over oöcyte development.

Shimizu and Hasegawa (1988) applied supplementary illumination to the heads of silk moth (*Bombyx mori*) larvae using a chemiluminescent paint whose major emission peak was close to 520 nm. Paint was applied to the entire head after the fourth instar and the treated larvae were then exposed to a range of photoperiods (LD 14:10, LD 15:9 and LD 16:8) spanning the critical day length. Emission from the paint declined rapidly after about 1 hour in darkness, so that effective photophases in the painted animals were extended. Results showed

an apparent shift of the critical day length to the left (shorter photophases) suggesting cephalic and probably brain photoreception.

(c) *Transplantation and in vitro illumination*

Using the giant silkmoth *Antheraea pernyi*, it proved possible to demonstrate that the photoperiodic receptors - and perhaps also the clock - were indeed in the brain (Williams, 1963; Williams and Adkisson, 1964). In a series of elegant experiments, Williams and Adkisson fitted diapausing pupae of *A. pernyi* into holes drilled in an opaque board in such a way that some of them received long-day illumination (LD 16:8) at their head ends and short-day illumination (LD 8:16) at their tails, and others the opposite combination. Out of eighty unchilled pupae maintained in such a 'photoperiodic gradient' (for 7 weeks), all of those with their heads exposed to long days developed and emerged as moths, whereas those with their heads exposed to short days remained firmly in diapause. The photoperiod that the tip of the abdomen 'saw' was inconsequential. The fact that the brain was the organ responsible for photoreception was demonstrated in a similar experiment in which brains were removed from twenty-six chilled pupae and then re-implanted under a plastic 'window' at the tip of the abdomen. These pupae were then placed in a photoperiodic gradient with a short day (LD 8:16) on one side and continuous darkness (DD) on the other. The results showed that ten (71 per cent) of the fourteen pupae with brainless anteriors exposed to LD 8:16 developed (i.e. a DD response), whereas none of the twelve pupae with 'brainy' abdomens exposed to LD 8:16 broke diapause. This experiment clearly demonstrated that transplantation of the brain to the tip of the abdomen also transplanted sensitivity to photoperiod, and thus identified the brain as the photoreceptor.

In principle the minimal mechanism for the photoperiodic response must include a photoreceptive pigment, a 'clock' to measure day length and to integrate photoperiodic 'information', and an output controlling development or diapause. In silkmoths it is known that the diapause control centres are in the brain, and that the neurosecretory cells in the *pars intercerebralis* synthesise and secrete brain hormone (PTTH) which, in turn, activates the prothoracic glands to produce ecdysone (Williams, 1952). Since the photoreceptors and the humoral effectors are located in the brain, it follows that the most likely site for the photoperiodic clock is also in the brain. It is interesting that the richest source of brain hormone in *A. pernyi* has proved to be the brains of diapausing pupae maintained in short day length (Williams, 1967). It would thus appear that short day length inhibits the translocation of the hormone along the neurosecretory axons, or its release from the neurohaemal organ, rather than its synthesis within the neurosecretory cells.

By a series of surgical procedures on the brains of diapausing *A. pernyi*, Williams (1969a) attempted to locate the photoperiodic mechanism within the brain itself. The strategy of these operations was to do as little as possible to the brain until its ability to differentiate between a long and a short photophase was finally destroyed. Each operation was performed on forty animals, twenty of which were then placed in a long day (LD 17:7) and twenty in a short day (LD 12:12). It was found that cutting the circum-oesophageal connectives, the tracheal connections to the brain, the nerves to the antennae or eyes, or the nerves to the *corpora cardiaca*, had no effect on the insects' ability to discriminate. Neither did bisection of the brain in the midline, or the dissociation of certain pigmented tips from the brain lobes. Only when the optic lobes were dissociated from the rest of the brain, or the entire brain excised and only the cerebral lobes re-implanted, was some damage to the mechanism evident. Even then, however, the insects could still discriminate between a short day (45 per cent reactivation) and

a long day (80 per cent reactivation). A similar result was obtained when only the dorsal half of the cerebral lobes was returned to the brainless pupa. Finally, when the whole brain was excised and only the *pars intercerebralis* (i.e. that part of the brain with the medial but not the lateral neurosecretory cells) replaced, the pupae then failed to respond to the opposing photoperiods. Williams pointed out that the proportion of these insects developing (25 per cent in both long and short days) was the same as in the LL control. He considered this a 'puzzling finding' because one might have anticipated that pupae deprived of their photoperiodic receptors would have behaved as if in constant darkness. No DD control was included, however, and the proportion of reactivating individuals in DD and LL are remarkably similar in this insect (Williams and Adkisson, 1964). Williams (1969a) concluded that "the photoperiodic mechanism is located in a tiny mass situated just lateral to the medial neurosecretory cells. Moreover, this crucial region includes the lateral neurosecretory cells."

Williams (1969a) attempted to dissociate the hormonal action of the neurosecretory cells from the electrical function of the surrounding neuropile by the use of puffer-fish tetrodotoxin which is thought to block the passage of sodium ions through electrically excitable membranes and therefore to block action potentials. When administered to pupae of *A. pernyi* at doses as high as 1 µg/g body weight the insects were paralysed but still underwent normal development to fully formed but flaccid moths which failed to escape from their pupae. More importantly, diapausing pupae of *A. pernyi* treated with tetrodotoxin were still able to discriminate between a long and a short photoperiod. Williams tentatively concluded that nerve impulses conducted in the neuropile played no part in the photoperiodic mechanism.

Claret (1966 a, b) also obtained evidence that the photoperiodic receptors lie in the brain. In the large cabbage white butterfly *Pieris brassicae*, the sensitive period is confined to a few days in the third and fourth larval instars. Claret transferred the brains from larvae just after the fourth moult, and therefore sensitive to photoperiod, into the abdomens of older larvae in which sensitivity had been lost. The recipients were then exposed to either short day (LD 8:16) or long day (LD 16:8) illumination. The results showed that those exposed to short days produced 60 per cent pupal diapause, whereas those exposed to long days produced only 23 per cent. It was concluded that the brain responded directly to the photoperiodic stimulus. In this connection it is interesting that the head capsule of the larva is entirely black before the third moult. After the third moult, however, the larva develops a translucent yellow triangle on the clypeus which admits light to the brain and sensitivity begins (Claret, 1966b). A similar change in the head capsule was reported for the silkworm *Bombyx mori* (Bounhiol and Moulinier, 1965) which is sensitive to photoperiod in its early larval development.

Conclusive evidence that the brain may contain the photoperiodic photoreceptors (and also the clock), however, requires *in vitro* illumination of excised brain complexes. This has been achieved in two species, the tobacco hornworm moth *Manduca sexta* and the commercial silk moth *Bombyx mori*.

M. sexta possesses a pupal diapause induced by short days perceived by the larvae; diapause may be averted by as few as three long day cycles during the final larval instar. Bowen et al. (1984) showed that a brain, together with *corpus cardiacum* (CC) and *corpus allatum* (CA), removed from such larvae and then implanted into the abdomen of a diapause-destined (short day) recipient, caused the latter to develop without diapause to the adult moth. They further showed that brain-retrocerebral (Br-CC-CA) complexes exposed, *in vitro*, to three cycles of LD 16:8 were capable of directing nondiapause development, whereas those similarly exposed to three cycles of LD 12:12 were not (Fig. 14.3). These experiments therefore demonstrated that excised brain-retrocerebral complexes could be photoperiodically 'programmed' *in vitro*. Such complexes respond to light, measure day length, store such

information, and then express it about nine days later, in the pupa, to regulate the release or retention of brain hormone (PTTH). These experiments thus demonstrated that both photoreceptor(s) and clock were brain centred, and were the first showing *in vitro* operation of a photoperiodic clock in any animal.

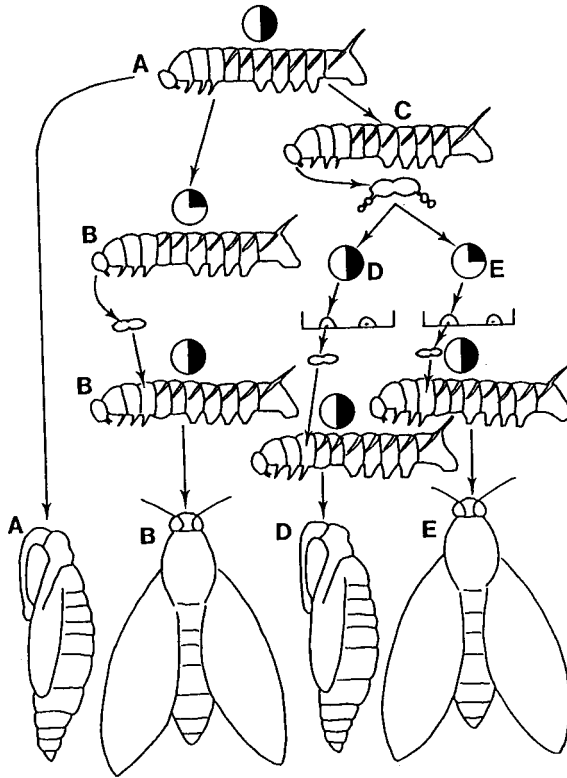


Fig. 14.3. *In vitro* operation of the photoperiodic clock in the tobacco hornworm, *Manduca sexta*. A – fifth stage larvae raised under short days (LD 12:12) give rise to diapausing pupae. B – larvae transferred to three long day cycles (LD 16:8) during the fifth instar, brain removed and implanted into the abdomen of a short-day (diapause induced) recipient larvae. This larva gives rise to a nondiapausing pupa and, subsequently, an adult moth. C – brains together with retrocerebral endocrine organs (corpora cardiaca, corpora allata) removed from short day larvae and exposed to either (D) three cycles of LD 12:12 in sterile organ culture, or to (E) three cycles of LD 16:8 in organ culture. These brains, now stripped of their cc and ca are implanted into recipient short-day (diapause programmed) larvae. D gives rise to diapausing pupae; E to nondiapausing pupae and emerging moths. (After Bowen et al., 1984).

These experiments with the hornworm were followed by similar *in vitro* studies using the silk moth *Bombyx mori* (Hasegawa and Shimizu, 1987). Brain-sub oesophageal ganglion (Br-SOG) complexes were removed from fifth stage larvae and exposed, *in vitro*, to four cycles of either long (LD 20:4) or short (LD 8:16) days before implantation into the abdomens of late fifth stage larvae. Results showed that implantation of short day complexes led to moths laying diapausing eggs whereas implantation of long day complexes gave rise to nondiapausing egg

producers. Since diapause hormone (DH) is secreted from the suboesophageal ganglion (Chapter 9, section B), cultured brains alone were ineffective. Like the earlier study with *M. sexta* these experiments showed that photoperiodic induction could occur *in vitro* and that the photoreceptors and the clock were located in the Br-SOG complex, most probably in the brain itself.

2. The role of the compound eyes

The first challenge to the widely held view that photoperiodic photoreceptors were entirely brain centred came from the work of Ferenz (1975) on testis development in the ground beetle, *Pterostichus nigrita*. In this species, long days suppressed maturation of the sperm. Bilateral extirpation of the eyes by cautery resulted in testis development as if the beetles were in continuous darkness, thereby suggesting that eye removal led to photoperiodic 'blindness'. It is of interest that such beetles have a very thick black cuticle over the head, probably rendering a more direct access to the brain unlikely.

This observation on *Pterostichus* was followed by a series of important papers by Numata and his colleagues who demonstrated optical photoreception in a number of insects including Heteroptera, Orthoptera and Diptera (reviewed by Numata et al., 1997). A variety of techniques to eliminate the eyes, or otherwise render them ineffective, were used. In the bean bug, *Riptortus clavatus*, a species exhibiting facultative ovarian diapause under short days, phosphorescent paint was applied to selected parts of the body. Bugs, some with and some without paint, were then maintained under three photoperiods (LD 12.5:11.5; LD 12.75:11.25; LD 13:11) just short of the critical value. The phosphorescent paint absorbed light energy and then for a while discharged its own green phosphorescence; painted bugs were therefore exposed to effective photophases slightly longer than the unpainted controls. Painting the compound eyes led to an increased proportion of the bugs developing their ovaries, as though they were indeed in longer days. Covering the vertex of the head capsule (over the brain) had no such effect. Numata and Hidaka (1987) then surgically removed the compound eyes of *R. clavatus* and showed that diapause termination under LD 14:10 was blocked. Removal of the ocelli had no such effect. These data strongly indicated that the compound eyes were the important photoperiodic photoreceptors. Morita and Numata (1997) later showed that photoreception was only eliminated when the central region of the eye was destroyed; the lateral regions were apparently not involved.

Working with the ground cricket *Pteronemobius nigrofasciatus*, a species showing maternal regulation of embryonic diapause under short days, Shiga and Numata (1996) showed that compound eye removal led to the production of nondiapause eggs, again suggesting ocular photoreception. Similar removal of the eyes, but not ocelli, of the stink bug *Plautia stali* (with a reproductive diapause) revealed developing gonads and enlarged *corpora allata* regardless of photoperiod (Morita and Numata, 1999). In this species, however, the response in male bugs was not totally eliminated by eye removal, perhaps suggesting some extra-optic photoreception, possibly in the brain itself. This raises the possibility of multiple light input pathways for photoperiodic induction (see below).

Although bilateral optic lobectomy in the blow fly *Calliphora vicina* (Saunders and Cymborowski, 1996) demonstrated that the compound eyes were not essential for photoperiodic induction, studies by Shiga and Numata (1997) on the closely related species *Protophormia terraenovae* indicated otherwise. In this blow fly, day length regulates an ovarian (reproductive) diapause in the adult fly. Shiga and Numata found that covering both of the compound eyes with silver paint led to an increase in diapause incidence under long days

or continuous light (intensity 0.5 lux), as if the flies were in continuous darkness. Silver paint applied to the dorsum of the head, or clear paint over the eyes, were both without effect. The apparent difference between *Protophormia* and *Calliphora* might be related to the comparatively opaque head cuticle in the former. Additionally it might be that *both* species use optic and extra-optic photoreception, the compound eyes becoming more important under lower light intensities. Once again these observations indicate multiple photoreceptive pathways, a probability that will be considered further.

3. Carotenoids and the role of vitamin A

The pronounced blue sensitivity revealed by action spectrum studies (see Chapter 10, G) suggested to many authors that the photoperiodic photoreceptor might contain a carotenoid based chromophore. Although some early attempts to demonstrate the importance of carotenoids were, at best, equivocal, later studies revealed such dependence in three species of mites and four insects.

Using albino mutants of the spider mite *Tetranychus urticae*, in which the uptake and metabolism of β -carotene was disturbed, Veerman and Helle (1978) and Veerman (1980) showed that the diapause response to short day length was lowered. Various crosses between albino and wild type showed that a high incidence of diapause was only realised when the albino daughters came from hybrid, phenotypically wild type mothers. In other words, a maternal effect was responsible for complete induction, and the albino locus was thought to exert a pleiotropic effect influencing both pigmentation and diapause. In a later paper, partial restoration of the short day response to such albino mites was shown to follow the addition of β -carotene to the diet, and a full restoration with added vitamin A (Bosse and Veerman, 1996). It was suggested that the photoperiodic receptor of *T. urticae* contained a vitamin A derived chromophore, possibly 3-hydroxyretinal. Restoration of function with vitamin A was greater than with β -carotene because the former was absorbed more easily.

In a series of elegant experiments, Veerman and his colleagues (Van Zon et al., 1981; Veerman et al., 1983; Overmeer et al., 1989; Van Houten and Veerman, 1990) demonstrated the importance of carotenoids and vitamin A in the photoperiodic responses of the predacious mites *Amblyseius potentillae* and *A. cucumeris*. Under short days, *A. potentillae* feeding on the eggs of wild type spider mites (*Tetranychus urticae*) entered a reproductive diapause. On the other hand, mites fed for several generations on eggs of albino mites – devoid of carotenoids – showed no such response (Van Zon et al., 1981). Mites raised on a diet of bean pollen were also unable to differentiate short from long days, but this response was restored following the addition to the diet of vitamin A, or of carotenoids with provitamin A function (Veerman et al., 1983). It was concluded that carotenoids or their derivatives were essential for the photoperiodic response, with a rhodopsin-like pigment functioning as the photoreceptor for diapause induction. However, in a particularly intriguing paper, Van Houten et al. (1987) showed that vitamin A was also required for *thermoperiodism* in *A. potentillae*. When mites were fed on bean pollen they were unable to differentiate between a 'short day' thermoperiod (8 hours at 27° and 16 hours at 15°C in DD) and a 'long day' thermoperiod (16 hours at 27° and 8 hours at 15°C in DD). They produced 5 per cent diapause or less in both regimes. After addition of β -carotene or vitamin A acetate to the diet, however, short day or diapause-inducing responses to the short thermoperiod were fully restored. This result seems to indicate that vitamin A might be involved in processes other than merely photoreception in this mite's diapause response.

Working with larvae of the south western corn borer, *Diatraea grandiosella*, raised on a carotenoid-free diet, Takeda (1978) found that diapause induction (short day LD 14.5:9.5 vs. long day LD 16:8) did not drop until the fifth generation, but was then restored within a further two generations after addition of β -carotene to the diet.

In the commercial silk moth *Bombyx mori*, Shimizu (1982) raised larvae on an artificial diet deficient in vitamin A. Such a diet rendered larvae visually 'blind', as measured by a loss of phototaxis and the ERG response. On the other hand, photoperiodic induction continued in these larvae - probably because sufficient vitamin A had been passed to the photoreceptor that required less for its normal function than the ocelli (stemmata). In a later paper, however, Shimizu and Kato (1984) showed that carotenoid depletion became evident after two or more generations on a diet without β -carotene, only 20 to 30 per cent of the larvae being able to respond to the diapause averting effects of a long day (LD 20:4). This response was almost fully restored by the addition of β -carotene to the larval diet. In an HPLC analysis of *Bombyx* brain tissues, Hasegawa and Shimizu (1988) subsequently demonstrated the presence of both retinal and 3-hydroxyretinal, known to be chromophores in insect visual systems. It was concluded that a retinoid protein probably functions as the photoperiodic receptor in this insect.

Claret (1989) raised larvae of the cabbage white butterfly, *Pieris brassicae*, on a carotenoid deficient diet. The first generation responded normally to photoperiod, but by the second generation the response to short days (LD 13:11, 25 lux), leading to pupal diapause, was severely reduced. Addition of vitamin A to the artificial diet then restored this response. In continuous darkness (DD) and a *thermoperiod* (8 hours at 21°, 16 hours at 13°C) diapause incidence was high in larvae reared on 'normal' (cabbage), carotenoid depleted, and vitamin A enriched diets. It was therefore concluded that vitamin A was essential for photoperiodic photoreception but not for thermoperiodism. In a later paper (Claret and Volkoff, 1992) vitamin A was also shown to be essential for the full appearance of 'point B' in 'night interruption' experiments, perhaps indicating photoreception at the putative 'photoinducible phase' (see Chapter 11, B1).

The fourth insect in which vitamin A has been shown to be essential is the parasitic wasp *Apanteles glomeratus* (Veerman et al., 1985). Larvae of this braconid develop as parasitoids within the larvae of their host, the cabbage butterfly *Pieris brassicae*. Under short days (<13 hours per day) the parasite larvae overwinter as prepupae within yellow cocoons outside the host's carcass; nondiapause larvae continue their development to the next generation within white cocoons. When the host larvae were reared on a carotenoid depleted diet, the wasp's photoperiodic response was absent. Addition of vitamin A to the caterpillar's diet, however, restored the wasp's response to short days indicating that vitamin A was essential for their photoperiodic responses. Since parasitoid eggs contain minimum yolk, little carotenoid was probably passed from one generation to the next, making *A. glomeratus* eminently suitable material for such an investigation.

4. Opsins and the phototransduction cascade

The nature of the photoperiodic photoreceptor and its anatomical location has been further investigated in the vetch aphid, *Megoura viciae*, by Gao et al. (1999) using immunocytochemical techniques. Twenty antibodies raised against various invertebrate and vertebrate opsins and proteins in the phototransduction cascade were used. Seven of these, including *Drosophila* rhodopsin 1, vertebrate cone and rod opsins, vertebrate arrestin, vertebrate transducin, and vertebrate cellular retinoid binding protein, consistently labelled an anterior ventral neuropile region of the protocerebrum, close to the medial (Group I) neurosecretory

cells considered to be the photoperiodic (hormonal) effectors. This region of the brain (Fig. 14.4), presumed to house the photoperiodic receptor, was also that revealed as important in earlier studies using supplementary illumination (Lees, 1964) and micro-lesions (Steel and Lees, 1977).

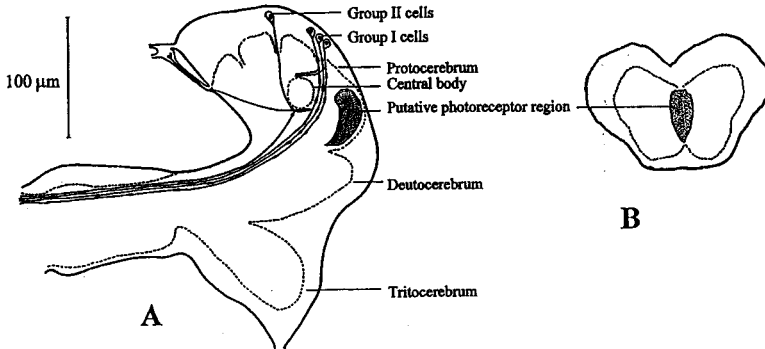


Fig. 14.4. *Megoura viciae*. A – sagittal section of the brain; B – vertical section, showing the position of the putative photoperiodic photoreceptors which lie in the inner neuropil region (dotted line) of the anterior ventral protocerebrum. The outer layer comprises the neuronal cell bodies, glial cells and perineurium (From Gao et al., 1999)

Foregoing sections (A to D) have outlined evidence that photoperiodic receptors may be retinal (compound eyes, but not ocelli) or extra-retinal (brain); in some species both may be important. Direct photic input to the clock via cryptochromes (see Chapter 4) is also possible. Therefore, as with overt circadian rhythms of behaviour (Chapter 8), *multiple* light input pathways for photoperiodic photoreception should perhaps be expected.

B. PHOTOPERIODIC CLOCK LOCATION AND OUTPUT

The pivotal event in diapause regulation is the release/retention of cerebral neuropeptides (PTTH, allatostatins/allatotropins etc.). Although we now have considerable knowledge of ‘downstream’ endocrine control of the diapause state (Saunders, 2000a), and are beginning to understand light input pathways to the clock, we remain woefully ignorant of the crucial events between photoreception and neurohormone release. Formal investigations of the clock and counter (Chapters 11 to 13) tell us that these events are functions of the circadian system and are almost certainly brain-centred; otherwise these central phenomena remain a ‘black box’. However, several recent studies on cerebral neurons using microanatomy and surgery, immunocytochemistry and molecular biological techniques may begin to provide concrete information on these processes.

1. Neurobiological studies

Several studies have directed attention to neurosecretory and other neurons within the insect brain. Working with the linden bug *Pyrrhocoris apterus*, Hodkova (1976, 1977) showed that cutting the *nervi allati* under long days (LD 18:6) did not affect ovarian development. The same operation under short days (LD 12:12), however, disturbed the inhibiting effects of short

days, bugs with cut nerves proceeding to lay eggs instead of maintaining ovarian diapause. Extirpation of the *pars intercerebralis* (PI) resulted in low reproduction under both photoperiods. It was concluded that the PI exerted both stimulatory and inhibitory effects on the *corpora allata* (CA). Under long days it stimulated the CA via the haemolymph and the glands responded by producing juvenile hormone. Under short days, however, the PI inhibited the CA by a neural channel through the *nervi allati*.

In the tobacco hornworm *Manduca sexta* (pupal diapause), Bollenbacher et al. (1993) demonstrated the presence of a pair of PTTH cells in the *pars lateralis* (PL) of each brain hemisphere. Intracellular recordings from these cells in diapausing pupae (Tomioka et al., 1995) showed them to become electrically 'silent' as diapause deepened, whereas those in nondiapause pupae showed significant spontaneous action potentials on day 2 of pupal development. It was tentatively suggested that the entire photoperiodic mechanism might reside in these cells. Evidence for this proposition was gained from the silk moth *Antheraea pernyi* in which tetrodotoxin failed to affect diapause termination and development of the moths under long days, thus indicating an endocrine rather than purely nervous regulation (Williams, 1969b).

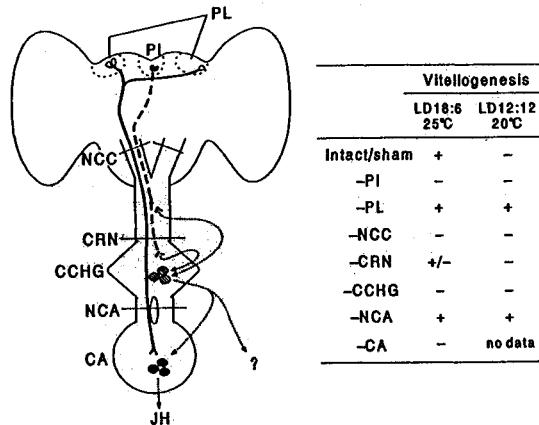


Fig. 14.5. *Protophormia terraenovae*. Effects of various surgical operations on ovarian development in adult flies. -PI, removal of the *pars intercerebralis*; -PL, removal of the *pars lateralis*; -NCC, transection of the *nervi corporis cardiaci*; -CRN, transection of the cardiac recurrent nerve; -CCHG, removal of the fused *corpus cardiacum* and hypocerebral ganglion; -NCA, transection of the *nervi corporis allati*; -CA, removal of the *corpus allatum*. + - vitellogenesis (nondiapause) observed in most females; - previtellogenic ovaries observed in most females. (From Shiga and Numata, 2000).

Recently, Shiga and Numata (2000) and Shiga et al. (2000) have examined the roles of neurosecretory neurons in the regulation of reproductive (ovarian) diapause in the black blow fly, *Protophormia terraenovae*. An anatomical study of these neurons by retrograde NiCl_2 filling through the cardiac-recurrent nerve revealed three groups of neurons - in the *pars intercerebralis* (PI), the *pars lateralis* (PL) and the sub-oesophageal ganglion. Retrograde filling from the corpus allatum (CA) revealed neurons in the PL, whereas most PI neurons led to the corpus cardiacum/hypocerebral ganglion complex. Microlesions to remove the PI in flies maintained under diapause inducing (LD 12:12, 20°C) and diapause averting (LD 18:6, 25°C) conditions led to ovaries that failed to develop under both conditions. However, when the PL was removed bilaterally, the ovaries developed in most females regardless of photoperiod. In

other words, neurons in the PI were necessary for vitellogenesis, whereas those in the PL were required for diapause induction under short days (Fig. 14.5).

Using immunocytochemical techniques, Sauman and Reppert (1996) mapped neurons of the silk moth *Antheraea pernyi* expressing the 'clock' proteins PER and TIM in relation to those expressing PTTH, eclosion hormone (EH) and pigment dispersing hormone (PDH). PER and TIM proteins were coexpressed in the cytoplasm and axons of eight cells in the dorsal lateral protocerebrum, the anatomical location of the circadian clock thought to drive rhythms of eclosion and adult flight (Truman, 1972b, 1974). These neurons were considered to be 'clock' cells even though evidence of a temporal movement of PER into the nucleus was lacking (see Chapter 4). There were no data on the possible role of these cells in photoperiodism and diapause regulation.

2. Melatonin as a photoperiodic clock output?

The indoleamine *melatonin* (*N*-acetyl-5-methoxytryptamine) plays several important roles in vertebrate circadian rhythmicity and photoperiodism (see Reiter, 1993). Melatonin-like activity also occurs across a wide range of other taxa, including the insects (Vivien-Roels et al. 1984; Finocchiaro et al., 1988). This suggests that melatonin is a highly conserved molecule although its role is often far from clear. In a number of insects, melatonin or its key enzymes show a clear rhythm with a maximum at night (Wetterberg et al., 1987; Tilden et al., 1994; L'Helias et al., 1995). For example, Itoh and Sumi (1998) recorded arylalkamine *N*-acetyltransferase (NAT)-like activity in the eggs of the cricket *Gryllus bimaculatus*; they showed that the rhythm peaked in the subjective night and free-ran in DD for about three cycles. In an earlier paper, Itoh et al. (1997) measured the titres of another key enzyme, hydroxy-*O*-methyltransferase (HIOMT) in the brains of fifth stage larvae of *Bombyx mori*. This enzyme also occurred in a nocturnal peak and free-ran in DD. The authors concluded: "The synthesis and release of melatonin occurred as a circadian rhythm that is entrained by light as it is in the vertebrate pineal. Melatonin may function as a neurochemical mediator of photoperiodic control."

A possible role for melatonin in photoperiodism has been investigated by applying it to insects under short nights in an attempt to prolong the naturally occurring nocturnal peak to mimic a *longer* night (or a shorter day). Hodkova (1989), for example, applied melatonin topically to the linden bug, *Pyrrhocoris apterus*, for four hours daily just before dusk. She found that such treatment delayed the onset of oviposition under LD 18:6 but did not induce diapause; neither did such treatment prevent termination of diapause in insects transferred from a diapause promoting LD 12:12 to a diapause averting long day. In a later study, Gao and Hardie (1997) fed pea aphids (*Acyrtosiphon pisum*) on an artificial diet containing melatonin. Under a long day regime (LD 16:8) and dietary melatonin their progeny were found to contain both males and virginoparous/oviparous intermediates, which normally only occur under short days or close to the critical night length. Radioimmunoassay revealed endogenous melatonin in this aphid, but the nocturnal peak was obscured by large variation. Gao and Hardie concluded that melatonin exists in the pea aphid, but that its role in photoperiodism was unclear.

Takeda et al. (1999) followed the dynamics of clock gene products, cyclic nucleotides, neurotransmitters and ecdysteroid titres in relation to the photoperiodic control of diapause and nondiapause pupal development of the silk moth, *Antheraea pernyi*. PER and *doubletime* (DBT) proteins were coexpressed in neurons considered to be the 'clock' cells. These cells also expressed arylalkylamine *N*-acetyltransferase (NAT) and hydroxyindol *O*-methyltransferase (HIOMT), enzymes in the melatonin biosynthetic pathway. Brain melatonin also fluctuated in

a circadian manner. The authors postulated that melatonin might be an output mediator of the photoperiodic clock in *Antheraea*, a proposition strengthened, perhaps, by the observation that melatonin has been found to regulate PTTH release in the cockroach *Periplaneta americana* (Richter et al., 1999). Although still highly speculative, this suggestion is important and warrants further critical consideration.

ANNOTATED SUMMARY

1. The light input pathway to the photoperiodic system frequently involves extraoptic or extraretinal photoreception in which neither compound eyes nor ocelli are involved. Direct photoreception by the brain is often indicated.
2. Brain photoreception has been confirmed by *in vitro* studies on *Manduca sexta* and *Bombyx mori* in which brains, receiving long or short days in organ culture, are transplanted into recipient insects to redirect their photoperiodic responses accordingly.
3. In an increasing number of insects, including beetles, crickets, hemipteran bugs and a fly, however, the compound eyes have been shown to be the relevant photoreceptor.
4. Carotenoid deficient diets may render insects (and mites) photoperiodically 'blind', whereas addition of β -carotene or vitamin A to such diets may restore the diapause response. It is concluded that the photoperiodic photoreceptor probably includes a retinoid protein as chromophore.
5. Immunocytochemical techniques using antibodies raised against a range of invertebrate and vertebrate opsins and proteins in the phototransduction cascade, have revealed probable photoreceptive sites in the aphid (*Megoura*) brain. The evidence on photoreceptive inputs suggests diverse and probably *multiple* light input pathways to the clock.
6. The photoperiodic clock is almost certainly brain-centred, with an output through neurosecretory neurons regulating neuropeptide (PTTH, allatotropin, allatostatin etc.) release/retention. Clock controlled PTTH cells are in the *pars lateralis* of *M. sexta* and *Protophormia terraenovae*.
7. The indolamine *melatonin* has been proposed as a photoperiodic clock output, although data supporting this contention are still equivocal. The proposition is strengthened by the observation that melatonin has been found to regulate PTTH release in *Periplaneta americana*.

This Page Intentionally Left Blank

CHAPTER 15

OTHER TYPES OF INSECT CLOCKS

And hand in hand, on the edge of the sand/They danced by the light of the moon.
Edward Lear

CONTENTS

Introduction	449
A. <i>The Time-memory (Zeitgedächtnis) of Bees</i>	449
B. <i>Time-compensated Sun Orientation</i>	453
C. <i>Semilunar, Lunar and Tidal Rhythms</i>	455
D. <i>Circannual Rhythms and 'Long-range' Timers</i>	466
E. <i>Ultradian Courtship Rhythms</i>	471
Annotated Summary	472

INTRODUCTION

SEVERAL types of clock are described in this chapter: each has a different and usually clear functional significance, and they show a variety of controlling mechanisms. The so-called 'time-sense' (*Zeitsinn*) or 'time-memory' (*Zeitgedächtnis*) of honeybees (Beling, 1929), and the time-compensated sun orientation of bees and other organisms (von Frisch, 1950; Kramer, 1950) are controlled by, or contain, an endogenous circadian component. The rhythm of emergence in the marine midges, *Clunio* spp. (Neumann, 1963, 1966 a, b) is controlled by a combination of endogenous circadian and *circasemilunar* rhythms entrained by the dominant environmental periodicities in their intertidal environment. There are also several long-period timers controlling the onset of diapause or seasonal morphs (Blake, 1958; Lees, 1960) which may be either rhythmic (*circannual*) or non-rhythmic in nature, but not clearly related to the circadian or hourglass-like timers involved in 'classical' photoperiodism. Short period (*ultradian*) rhythms such as the male courtship song rhythm of *Drosophila melanogaster* (Kyriacou and Hall, 1980) are also briefly reviewed.

A. THE TIME MEMORY (*ZEITGEDÄCHTNIS*) OF BEES

The ability of honey-bees (*Apis mellifera*) to return to a food source at the same time each day has been known since the turn of the century when the Swiss naturalist August Forel observed bees arriving at his breakfast table for food. Since they always came at the same time - even when food was not present - Forel (1910) proposed that the bees had a 'memory' for time (*Zeitgedächtnis*). Similarly, von Buttel-Reepen (1900) observed that bees only visited a

buckwheat field in the morning when the blossoms were secreting nectar; he concluded, therefore, that the insects possessed a 'time-sense' (*Zeitsinn*).

Modern work on the bees' *Zeitgedächtnis* began with Beling in 1929. She trained bees to an artificial feeding place by offering sugar solution at the same time each day. Individual bees were marked with paint when they were feeding on the sugar. During subsequent days (the test period) the feeding place was without sugar, but each visiting bee and its time of arrival was recorded. Beling demonstrated that bees do indeed come back at the same time each day (Fig. 15.1). She also showed that bees could be 'trained' to come at any time of the day and, moreover, that they could be trained to come at two or more separate periods during the day provided that the interval between two successive training periods was greater than two hours.

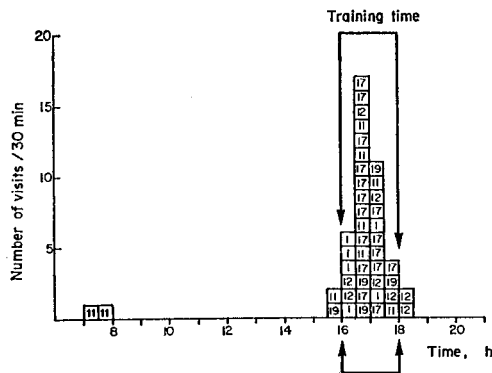


Fig. 15.1. The time-memory (*Zeitgedächtnis*) of bees. The bees were 'trained' to come to a sugar source at a fixed feeding position at the same time (16 to 18 hours) during several consecutive training days. As they visited the sugar they were marked individually. On the 'test day' the sugar was omitted, but the bees continued to arrive at the dish at the same time of the day. The numbers refer to the individually marked bees. (After Beling, 1929.)

These experiments confirmed the earlier observations of Forel and von Buttel-Reepen but did not answer the question whether the bees possessed an innate 'time memory' or 'clock', or whether they were merely responding to external signals such as the position of the sun in the sky. Beling (1929), however, also conducted an experiment in which obvious time cues, such as the daily cycles of light, temperature and humidity, were removed. Subsequently, Wahl (1932) took elaborate procedures to exclude even cosmic radiation by conducting the entire experiment in a salt mine 150 m below the surface of the earth. In both experiments the bees returned punctually to the feeding place during the test period, indicating the endogenous nature of the clock involved. Beling (1929) and Wahl (1932) also showed that it was impossible, even after weeks of training, to train bees to a feeding rhythm too far removed from that of the solar day. Attempts to train bees to a 48-hour rhythm, for example, resulted in foraging activity every 24 hours (Beling, 1929). It seemed, therefore, that the bees' time sense was - in modern terminology - controlled by an endogenous circadian oscillation.

The unequivocal test for endogeneity, however, was not performed until the 1950s. Renner (1955, 1957), using essentially the same technique as Beling, trained bees to a food source in Paris (2°E) between 8.15 and 10.15 local time in a closed chamber in constant light (LL) and constant temperature. The bees were then transported overnight to New York (74°W)

and tested the following day under identical conditions. In this now classical 'translocation' experiment, the bees had been transported over 76° of longitude, or a difference of about 5 hours in real local time. If an endogenous circadian rhythm was involved the bees should have come to the test dish 24 hours after the training period; if, on the other hand, the bees were responding to subtle local influences, they should forage at the same local or sun time. The results showed that the former alternative was the case: the bees came to the feeding dish at 3.00 Eastern daylight time, exactly 24 hours after their last feeding period in Paris. The reciprocal experiment involving a translocation from New York to Paris had an analogous result.

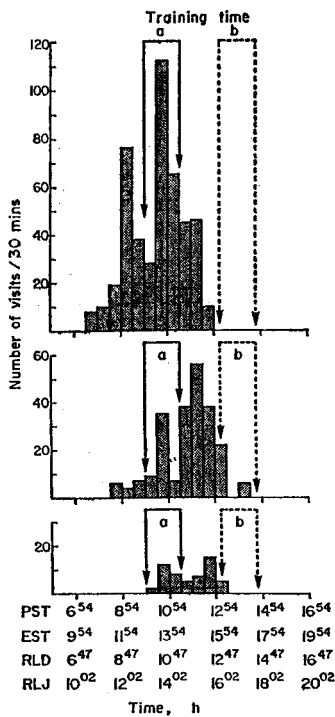


Fig. 15.2. Visiting frequency of bees which were trained in the open air to a feeding time of 12.54 - 2.54 p.m. EST at St. James (Long Island, N.Y.) and which were tested after an overnight translocation over 49° longitude to Davis, California. The three panels show the visiting frequency of the bees on three consecutive test days at Davis. a - 24-hour term of the training period, b - day time at Davis which corresponds to training day time at St. James. *Abscissa*: PST Pacific Standard Time, EST Eastern Standard Time, RLD Real local time at Davis, RLDJ Real local time at St. James. *Ordinate*: number of photoelectrically induced recording impulses caused by the bees looking for food at the recording boxes. (Redrawn, after Renner, 1960.)

This experiment was subsequently repeated in the open. Renner (1959) trained bees to a sugar source in a field on Long Island, N.Y., between 12.54 and 2.54 p.m. Eastern Standard Time. They were then flown overnight to Davis, California (a change in longitude of 49° , and a difference in real local time of 3 hours 15 minutes), and tested in the open on successive days.

The results showed that foraging activity occurred initially at a time 24 hours after the last feeding period on Long Island, but then showed signs of re-entrainment to the local light-cycle by the third day (Fig. 15.2). This behaviour, therefore, was very similar to that of endogenous activity rhythms subjected to similar phase-shift experiments (Chapters 2 and 3). Further evidence for the endogenous and circadian nature of the bees' *Zeitgedächtnis* was obtained by Bennett and Renner (1963), Beier (1968) and Beier and Lindauer (1970) who demonstrated that the rhythm free-ran in LL with a natural period (τ) of 23.4 to 23.8 hours. In addition, despite the failure of earlier workers to entrain the bees to cycles other than 24 hours (Beling, 1929; Wahl, 1932), they showed that entrainment was possible to LD cycles between 20 and 26 hours. The primary range of entrainment was therefore similar to other circadian oscillations.

A number of authors attempted to influence the *Zeitgedächtnis* clock by changing the bees' metabolism (Wahl, 1932; Grabensberger, 1934; Kalmus, 1934; Werner, 1954; Renner, 1957). These experiments showed that increasing the rate of metabolism with thyroxine, or decreasing the rate of metabolism with quinine, had no effect on the time-sense. Chilling to 4 or 5°C for about 5 hours, however, caused a 3 to 5-hour delay in their arrival at the test feeding dishes. After CO₂-narcosis (Medugorac, 1967; Medugorac and Lindauer, 1967) the bees turned up at the original feeding time *and* some time later, the delay of the second peak depending on the duration of narcosis and on the concentration of the CO₂. Medugorac and Lindauer (1967) concluded that at least two clocks were involved in *Zeitgedächtnis*, one that could be delayed by CO₂ narcosis, the other which could not. Hoffmann (1971) pointed out that this result, and its interpretation, was consistent with the multioscillator concept developed for the clocks controlling activity rhythms and for photoperiodism (see Chapter 6).

The 'time-memory' of bees therefore depends on endogenous circadian rhythms possessing properties common to all circadian systems. Since bees can be trained to come to a food source at *any* time of the day, or to more than one such time, *Zeitgedächtnis* comes under Pittendrigh's (1958) designation of a 'continuously consulted' clock. It is also probably of Truman's (1971) type-II clock because of its obvious affinities to an activity rhythm. The adaptive significance of this type of clock is obvious: it enables bees to return to a known food source when nectar and pollen are most readily available (Kleber, 1935) and therefore to maximise productivity. One advantage of the clock being oscillatory is that bees can stay in the hive for a day or two because of bad weather and still 'remember' the time when a particular plant species secretes its nectar. The fact that the rhythm is fairly easily extinguished without positive reinforcement, however, is also of biological importance because there is an ever-changing array of nectar sources, and there is little selective advantage in continuing to arrive at flowers long past their best.

In a more recent and very thorough investigation of the bees' *Zeitgedächtnis*, Moore et al. (1989) confirmed the major features of the phenomenon. They also showed that the temporal accuracy of foraging visits varied across the day. It was nearly exact for bees trained to forage in the morning, but less so for those trained later in the day. This daily change in accuracy was considered to be an endogenous (circadian) behaviour pattern. Two separate processes were thought to contribute to *Zeitgedächtnis*. The first varied with the time of day and determined the amount of anticipatory activity directed towards the food source. The second process was invariant across the day and was involved with the individual's continuous and accurate estimation of time.

Two papers claimed transplantation of the *Zeitgedächtnis* mechanism. Martin et al. (1978) trained 'donor' bees to feed for five consecutive days from 2000 to 2200 hours under conditions of constant light. 'Recipient' bees were similarly trained, but from 0600 to 1100

hours. The donor bees' mushroom bodies were then removed and inserted into the heads of the recipients (which still retained their *own* mushroom bodies). Over the next five days the feeding behaviour of the recipients was followed. On days 1 and 2 there was apparently no clear timing to their feeding, but on days 3 and 4 a peak in the recipients' foraging activity occurred about 1 hour after the *donors'* training time. The authors concluded that transfer of "the time signal" had been accomplished, thereby suggesting that the mushroom bodies were the anatomical sites of the *Zeitgedächtnis* 'mechanism'. In the second paper, Martin and Martin (1987) described an experiment resembling Renner's classical 'translocation' experiment (see above). Thus donors were trained to feed for 4 days at about 1300 hours local time in Seattle, U.S.A. Mushroom bodies were then extracted, placed in liquid nitrogen, and flown to Wurtzburg, Germany. Here - after *two months* storage in liquid nitrogen - they were implanted into recipient bees offered food continuously in an experimental flight room under constant light. When the recipients' foraging activity was examined, it was reported to show, by day 5, a peak of feeding corresponding to 1300h Wurtzburg time and not 1300 h Seattle time. Consequently it was claimed that the bees' *Zeitgedächtnis* was not controlled by an endogenous, or circadian, clock, but was an exogenous response to the local geomagnetic field. The authors thus reopened an old controversy, long since settled, as to whether circadian rhythms were exogenous responses to 'subtle geophysical variables', or were the overt manifestations of endogenous, physiological pacemakers (see Chapter 1).

Brady (1987), however, provided an excellent critical re-examination of these two papers. Whilst accepting that bees might respond to the earth's magnetic field, this signal was more likely to act as a *Zeitgeber* than a *causal* influence on rhythmicity. Bees, like all other organisms, show clear free-running circadian rhythms under constant light with a natural period (τ) of less than 24 hours (see for example Spangler, 1972; Moore and Rankin, 1985; Frisch and Aschoff, 1987), amply demonstrating the endogenous nature of such rhythmicity. It was considered extraordinary that transplantation of mushroom bodies should be effective after two months in liquid nitrogen, and even when they were partially decomposed. Brady also drew attention to a lack of suitable controls: no sham-operated bees or unoperated Wurtzburg recipients. In a re-examination of the Martins' 'translocation' experiment it was further shown that initial post-operative feeding peaks were apparently closer to Seattle's 1300 h feeding time than to Wurtzburg's. Whether this implied transfer of phase or merely the restarting of the bees' own clocks, however, was impossible to determine given the lack of suitable controls. It was concluded that the data were consistent with the accepted explanation of endogenous circadian rhythmicity. The data contained no evidence for exogenous responses to local geophysical variables, or for the transplantation of such an exogenous response with the bees' mushroom bodies.

B. TIME-COMPENSATED SUN ORIENTATION

A number of insect species are known to orientate themselves by maintaining a fixed angle to the sun, to an artificial light source, or to the pattern of polarised light from a blue sky. Santschi (1911, 1913), for example, showed that ants used the position of the sun (the 'light compass reaction') to maintain a straight course in territory poor in landmarks. He also showed that if the ants were shielded from the direct rays of the sun, but exposed to its reflection in a mirror, they altered their course in a predictable fashion. The sun, of course, 'moves' during the day, and if the light-compass reaction is to be at all useful for longer-term orientation, the animals must be able to compensate for such changes in the sun's azimuth. Early experiments by Brun (1914) with the ant *Lasius niger* seemed to discount this possibility.

He showed that if ants were detained for a few hours in a darkened box and then released, they continued at the *same* angle to the sun and therefore in a different compass direction (but see the work of Jander, 1957, below). Wolf (1927) demonstrated light-compass orientation in the honeybee but also found no evidence to suggest that the insects could compensate for the sun's motion.

The fact that some organisms possess an innate biological clock for such time-compensation, however, was demonstrated almost simultaneously and independently by von Frisch (1950) for the honey-bee and by Kramer (1950) for the starling. Von Frisch trained bees to visit a feeding place west of the hive in the evening, then moved the hive during the night to a new site in unfamiliar surroundings. When the bees' behaviour was tested the next morning they were found to forage to the west, even though they now had to fly away from the sun instead of towards it as in training. von Frisch and Lindauer (1954) later conducted a similar experiment. Bees were trained to visit a sugar source 180m north-west of the hive and the whole colony was then moved overnight to new and unknown territory. Next morning the bees were offered four feeding choices each placed at the same distance (180m) from the hive but at different compass directions (NE, SE, SW and NW). Despite the fact that there were no familiar landmarks and that the sun was now in the east instead of the west, the majority of the bees came to the feeding table in the training direction, i.e. to the north-west (Fig.15.3). Subsequently Meder (1958) trained bees to a certain direction from the hive, then captured them at the feeding table and kept them for one or more hours in the dark. When they were released they flew unerringly in the direction they had been trained despite the fact that the sun had 'moved' during the bees' captivity. These experiments all indicated that bees are able to compensate for the movement of the sun allowing them to maintain a constant compass direction.

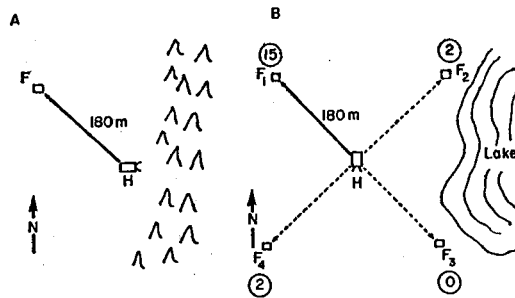


Fig. 15.3. Time-compensated sun orientation in the honey bee. A - a beehive was placed in an unknown region, and a group of bees was fed in the afternoon on a feeding table 180 m NW. B - during the night the hive was moved to another area and in the morning the bees had to choose one of four feeding tables 180 m NE, NW, SE or SW of the hive. The new landscape did not offer any familiar landmarks; the sun stood at another angle relative to the training line as in the previous afternoon. Nevertheless, most bees (encircled numbers) came to the NW, i.e. bees had calculated the sun's movement. (After Lindauer, 1960.)

To an observer (or to a bee) in the northern hemisphere the sun appears to 'move' in a clockwise direction from east to west, whereas in the southern hemisphere its movement appears to be anticlockwise. Bees transported from the northern to the southern hemisphere, or *vice versa*, therefore, are confronted with the task of orientating to sun movements in an apparently opposite direction to that in their original home. Transportation experiments of this

nature, designed to test whether the time-compensated sun-orientation behaviour in bees is inherited or learned, have produced equivocal results, however. Kalmus (1956) moved bees from North America to Brazil and studied the orientation behaviour of their descendants. He found that the bees continued to orientate themselves as though the sun was still moving in a clockwise direction. Lindauer (1957) in a repeat of Kalmus' experiment, however, found that the F_1 progeny of bees moved from North America to Brazil orientated correctly to southern hemisphere conditions. Transportation of a colony of *Apis indica* from Ceylon to Germany caused initial disorientation but, after 43 days, bees trained to the south in the afternoon were able to find correct direction the following morning after an overnight removal to unfamiliar territory (Lindauer, 1959). Lindauer (1960) concluded that the time-compensated orientation mechanism was innate, but the bees had to learn the (apparent) direction in which the sun moves, and its 'speed'. Edrich (1981) has more recently addressed the problem of bees' sun-compass behaviour at the equator.

The use of a clock to compensate for the changing azimuth of the sun has also been described for ants (Jander, 1957), the beetle *Geotrupes sylvaticus* (Birukow, 1953; Geisler, 1961) and for the pond skater *Velia currens* (Birukow, 1956; Birukow and Busch, 1957). Jander (1957) found that ants (*Lasius niger*) continued with the same compass direction after being confined in a dark box, showing that they had taken into account the sun's movement. This result, therefore, was contrary to that of Brun (1914) mentioned above. A similar result was obtained for *Formica rufa*, but only during the summer: in March and April this species was apparently unable to compensate for solar movement and showed an incorrect compass direction after being imprisoned in the dark (von Frisch, 1967, p. 448). Evidently compensation for the changing azimuth has to be learned anew after the winter.

The pond skater *Velia currens* is apparently able to orientate to an artificial light source, to the sun or to the plane of vibration of polarised light from a blue sky. According to Birukow (1960), the insects ran exactly southwards when placed on dry ground under a blue sky, and compensated for the shifting position of the sun during the day. Figure 15.4 shows that the angle of orientation to the sun or to an artificial light source decreased from sunrise to noon on the insect's left side and then increased on the right to sunset. From sunset to midnight the angle decreased on the animal's right side and then from midnight to sunrise it increased again on the left. It appeared, therefore, that the underlying process ran through the night but in the opposite direction. The *Velia* 'clock' appeared to show some characteristics of an oscillatory system such as dependence on photoperiod and re-entrainment to a reversed light-cycle. However, the animals tended to move directly to the light source after about 30 hours in LL or DD, casting doubt on the existence of a persistent endogenous periodicity. Birukow (1960) considered that the orientational clock in *Velia* was mainly regulated by exogenous light signals. In later experiments with this species, Heran (1962) was unable to find any compass-true southerly course, and in the open the insects were found to orientate preferentially in relation to the wind. Further examination of orientation in *Velia* would therefore seem to be necessary.

C. SEMILUNAR, LUNAR AND TIDAL RHYTHMS

Semilunar Rhythms in Marine Midges

Midges of the genus *Clunio* (Chironomidae) are found in the intertidal zones of Atlantic and Pacific shores from temperate areas to the Arctic. They have - for insects - very curious life cycles. *Clunio marinus* on the Atlantic coast of western Europe, for example, lives in the

lowest parts of the intertidal zone which are only exposed during the times of spring low water. It is only during these times that the insects are able to emerge (Neumann, 1963). The females of this species are wingless. The males emerge before the females, assist the females in their own emergence, copulate with them and then carry them to the larval habitat. Both sexes are extremely short-lived (~2 hours) and oviposition must occur before the tide rises to cover the larval site.

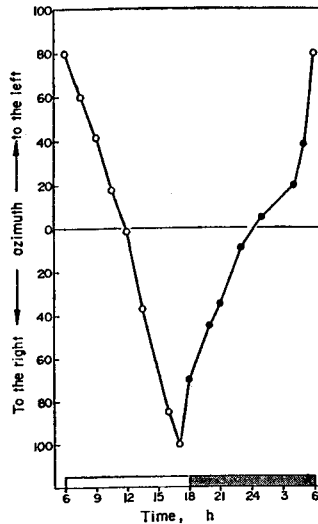


Fig. 15.4. Mean angles of orientation to an artificial light source of ten specimens of *Velia currens* in ten successive trials during 'day' and 'night' of an LD 12:12 regime. Ordinate: angles of orientation. Abscissa: clock readings in central European time. (Redrawn, after Birukow, 1960.)

On the Normandy coast low water occurs twice a day at intervals of 12.4 hours; consequently low and high tides are about 50 minutes later each day and it takes a period of about 15 days before the times of low and high water complete a full cycle. Superimposed on this semidiurnal tidal cycle is a semilunar tidal range. Tides reach their lowest point (spring low water) twice during each lunar month, once just after the full moon and once just after the new moon. Neap tides also occur twice per lunar month and occur just after half moon. The period between successive low waters is 14.77 days (Fig. 15.5). On the days of the spring tides, low water occurs in the early morning and again in the evening. Emergence of *C. marinus* in this locality is restricted to the evening low water, just following full and new moon. The insect therefore shows a well-marked diurnal (= circadian) and semilunar (= circasyzygic) rhythm of eclosion (see review by Neumann, 1976a).

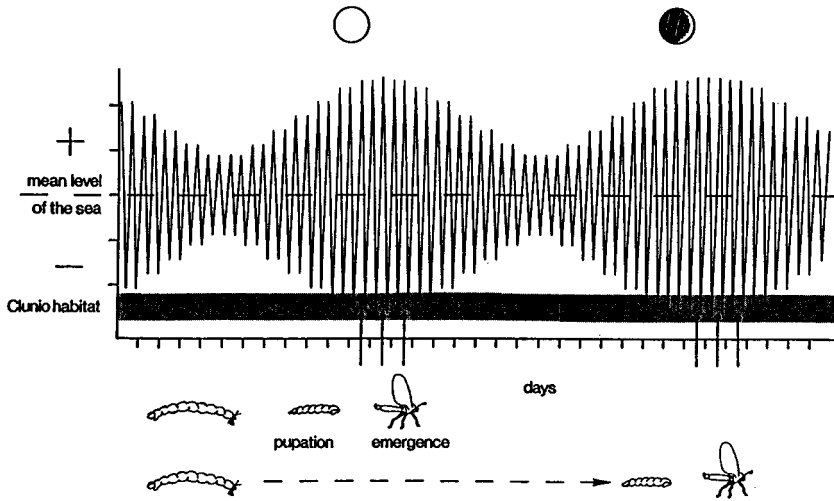


Fig. 15.5. The emergence of the marine midge *Clunio marinus* in natural tidal cycles, showing its semilunar periodicity. Upper panel: Semilunar changes in tidal heights correlated with phases of the moon. The *Clunio* larval habitat is only exposed for a few days during the lowest spring tides; eclosion occurs during the afternoon low waters. Below: the life-cycle of *C. marinus* correlated with the tidal cycle. (From Neumann, 1976b.)

In a series of elegant experiments, Neumann (1963, 1966 a, b) analysed the rhythm of adult emergence in *C. marinus* and showed it to be governed by the superposition of a circadian rhythm controlling pupal eclosion and a semilunar rhythm determining the beginning of pupation. In populations of *C. marinus* reared in the laboratory, eclosion occurred towards the end of the photophase (i.e. about 12 hours after light-on in LD 16:8), thereby corresponding to the observed time in the natural habitat. Cultures raised in LL throughout development showed an arrhythmic pattern of eclosion, but transfer from LD to LL, or exposure of an LL-raised culture to a single dark period, initiated a rhythm of eclosion which free-ran with an endogenous periodicity (τ) of less than 24 hours (Fig.15.6). These experiments clearly show that pupal eclosion is controlled by a circadian clock similar to that described for *Drosophila pseudoobscura* (see Chapter 3).

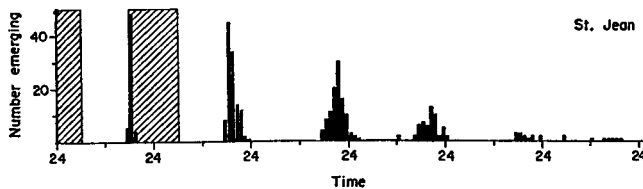


Fig. 15.6. A population of *Clunio marinus* from St. Jean-de-Luz released from LD 12:12 into constant light (LL) showing the free-running rhythm of pupal eclosion. (From Pflüger and Neumann, 1971.)

The semilunar rhythm of pupation which is superimposed on the circadian cycle was shown to be entrained by natural or artificial moonlight (Neumann, 1966b). Cultures of *C. marinus* from Normandy were raised in LD 12:12 or LD 16:8 and then exposed to pulses of

weak nocturnal light (0.4 lux) during the dark period for 4 to 6 days at intervals of 30 days. This treatment initiated and entrained a semilunar rhythmicity (Fig. 15.7) which was absent from control populations without artificial moonlight. The endogeneity of this rhythm was demonstrated by exposing a population of larvae to a single period of artificial moonlight, whereupon the system 'free-ran' for more than three cycles (each of about 15 days) before all the individuals in the population had completed their development.

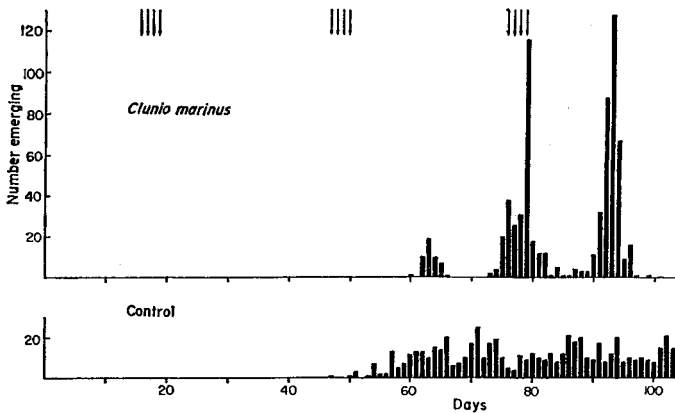


Fig. 15.7. Semilunar rhythm of emergence in *Clunio marinus* induced by artificial moonlight (4 nights with weak - 0.4 lux - light every 30 days) in a population from Normandy, France. Above: experimentals; below: controls without additional illumination at night. (From Neumann, 1966b.)

Neumann (1976b) examined the possibility that this semilunar rhythmicity was a consequence of the 'beat' hypothesis - a 24 hour circadian rhythm interacting with a 12.4 hour tidal component - to produce a period of about 14.7 days. This was done by raising populations of *C. marinus* in light-cycles of LD 11.6:11.6 ($T = 23.2$ hours) or LD 12.2:12.2 ($T = 24.4$ hours) which would be expected to entrain the circadian rhythm but leave the tidal component free-running. The interaction between $T = 23.2$ hours and the tidal rhythm should then, according to the 'beat' hypothesis (Bünning, 1973), have resulted in peaks of eclosion every 8 to 9 days, whereas an interaction between $T = 24.4$ hours and the tidal component should have produced peaks every 30 days. Results, however, showed no change in the semilunar periodicity, suggesting that it is a true long-term endogenous system not generated by such an interaction. This was further supported by a lack of change in the semilunar period following 4-hour advances or delays in the light-cycle.

Considerable differences are known to occur between populations of *C. marinus* in different localities, and between different species of the genus. The first of these arises because, although the dates of spring tides are the same at all parts of the same coastline, the phase of the 12.4 hour tidal cycle relative to local time shows differences, even at the same longitude. For this reason different local populations of *C. marinus* show different emergence times. These differences, moreover, have been shown to be genotypic (Neumann, 1966 a, b). Figure 15.8 shows the results of cross breeding experiments between populations from Normandy (Port-en-Bessin) and the Basque coast (St. Jean-de-Luz). In the natural populations emergence occurs between 1400 and 1600 hours in Normandy and between 1800 and 2000 hours in the south. Because of the extremely short-lived nature of *Clunio* adults, experimental

cross breeding could only be achieved by manipulating the light-cycles so that the emergence times coincided. The results of such crosses showed that the emergence times of the F_1 and the F_2 progeny were strictly intermediate between the parental times, although the 'spread' of emergence was greater for the F_2 . Neumann (1966b, 1967) concluded that eclosion time was controlled by a polygenic mechanism, but that a small number of genes were involved. Results for back crosses between F_1 and Normandy parents were consistent with this view. Between some local populations of *C. marinus* a non-reciprocal cross sterility was found (Neumann, 1971). The cross between St. Jean-de-Luz females and Santander males, for example, was fertile, but the reciprocal cross (Santander females x St. Jean-de-Luz males) was not. This unilateral crossing ability is thought to represent a specialised mechanism that in natural populations would be effective in the formation of physiological races.

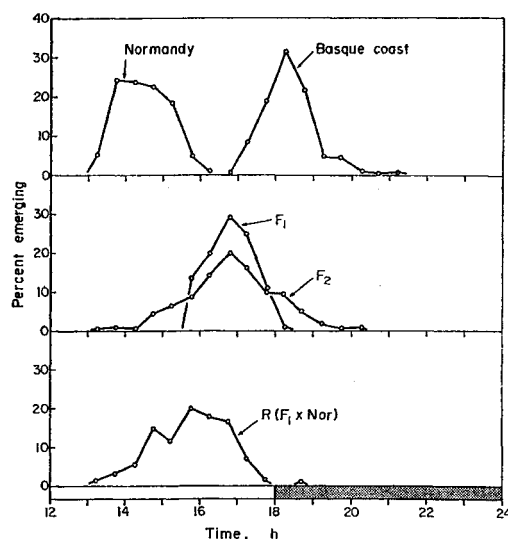


Fig. 15.8. Cross-breeding between two stocks of *Clunio marinus* which differed in their diurnal emergence times. Above: diurnal emergence times of the parental stocks Normandy (Port-en-Bessin) and Basque Coast (St. Jean-de-Luz). Middle: F_1 and F_2 generations. Below: backcrosses between F_1 and Normandy stock (in all curves only the emergence times of the males are presented). Breeding conditions: LD 12:12, light from 6 to 18 hours, 20 °C. (From Neumann, 1967.)

In a more northerly population of *C. marinus* from Helgoland (54°N), Neumann (1966b) found only weak entrainment with artificial moonlight. At this latitude the moon may not be bright enough: its maximum 'altitude' is, on average, only 12.5°, and the shorter summer nights are not so dark as those further south. In this population, however, it was found that the semilunar cycle could be entrained by tidal stimulation. A similarly behaving population from the English Channel coast near the Isle of Wight occurred in an area where the semidiurnal tidal curves are distorted, times of low water varying widely along quite short stretches of the coast. In response to this, a much wider, and therefore more 'flexible', gate has evolved (Heimbach, 1978a).

In arctic populations of *C. marinus* from Tromsø, Norway, there was a strictly tidal (~12.4 hour) cycle of eclosion in the summer (Neumann and Honegger, 1969; Pflüger and Neumann,

1971), emergence time coinciding with the initial exposure of the larval habitat during each ebb tide. Pflüger and Neumann (1971) showed that populations raised in LD 16:8 emerged as adults about 10 to 11 hours after light on, but when they were 'released' into LL no persistent rhythm could be detected (Fig. 15.9). In conditions of continuous light interrupted by a single 6 hour dark period, or in DD interrupted by a single 6 hour light pulse, a single peak of eclosion occurred about 10 to 11 hours after light-on. It was concluded that an hourglass-like mechanism starting at least 10 to 11 hours earlier during the preceding ebb, rather than a free running circadian oscillator, was involved in this Tromsø population. The 'hourglass' could be, of course, a heavily damped oscillation (see Chapter 7).

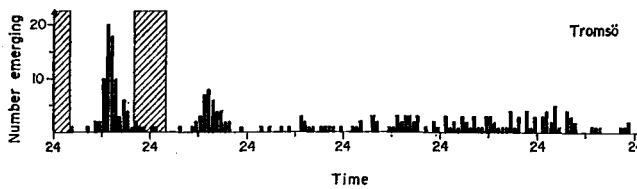


Fig. 15.9. A population of *Clunio marinus* from Tromsø, Norway, released from LD 16:8 into constant light (LL) showing one further emergence peak and then arrhythmicity. (From Pflüger and Neumann, 1971.)

Alternative patterns of eclosion are to be seen in other marine midges. The Japanese species *C. takahashii*, for example, is restricted to the mean intertidal level and is therefore exposed twice daily by the tidal cycle throughout the season; emergence occurs on every ebb tide (Hashimoto, 1966) as with the arctic population of *C. marinus*. Populations of *C. mediterraneus* from Yugoslavia inhabit a coast with a small tidal range, but a diurnal cycle of land- and sea breezes. During the summer months a relatively high fall of the tide occurs between midnight and sunrise during the strongest influence of the land breeze. Every 15 days, following both full and new moon, the lowest tides coincide with the times of the strongest land-breezes; larval habitats are then exposed and eclosion occurs (Neumann, 1967). Lastly, the marine chironomid *Thalassomyia frauenfeldi* shows quite a different eclosion strategy (Neumann et al., 1997). Unlike *C. marinus*, *T. frauenfeldi* has a life span of several days during which time the adult females walk on exposed intertidal substrates apparently waiting for favourable low water levels. Eclosion in this species, therefore, shows wide diurnal gates and no semilunar rhythmicity, even though it occupies similar niches to *C. marinus* and occurs in the same localities.

The *Clunio* population in the tideless Baltic differs from *C. marinus* of the North Sea and Atlantic coasts and is regarded as a distinct species, *C. balticus* (Endrass, 1976). The larvae of this species live on the bottom in permanently submerged habitats, and adult eclosion shows no semilunar rhythmicity. The eggs also differ from *C. marinus* in possessing a gelatinous matrix which swells after the eggs are laid on the water surface; the eggs then sink to the bottom. *C. balticus* also shows two distinct generations per year, one in the spring, the other in the summer, with the spring generation developing from larvae which overwinter in a state of photoperiodic diapause (see Chapter 9, A), induced in the laboratory by short days (LD 8:16) and terminated by long days (LD 16:8).

Heimbach (1978b) found *C. balticus* and *C. marinus* occurring as sympatric species near Bergen, Norway. As in the Helgoland population, *C. marinus* was intertidal and emerged in the afternoon during low spring waters with a pronounced semilunar rhythm. *C. balticus*, on the other hand, occurred as a sub-littoral population emerging nearly every day during the summer

months just after sunset and independently of the tides. In their natural habitat *marinus* was found to emerge about 4 to 5 hours before *balticus*. Laboratory-bred hybrids were found to have an intermediate emergence time, but these hybrid times were never observed in the field, suggesting a complete genetic isolation between closely related sympatric species on the basis of their eclosion times.

During the 1980s and 1990s further work by Neumann and his colleagues established the semilunar rhythm in *Clunio* spp. as one of the best-known examples of an endogenous 'circa-rhythm' in the whole of chronobiology. These observations included data on the influence of tidal temperature cycles, the range of entrainment of the circadian component, temperature compensation of the circa-semilunar period, developmental observations on the semilunar 'switching point', and on the photoperiodic regulation of larval diapause. These advances will be expanded below.

Neumann and Heimbach (1984) found that tidal temperature cycles combined with daily light cycles could interact as a *Zeitgeber* regulating the semilunar rhythm of *C. marinus* because the two cycles attained a particular mutual phase relationship every 15 days. Experimentally, two types of temperature cycles were used: sinusoidal fluctuations and short term 'pulses' (1.5 hours of raised temperature, 3 to 5°C amplitude) repeated every 12.4 hours to mimic the changes of temperature associated with inundation and exposure during the natural tidal cycle. Results showed that the end of the warming phase was a decisive parameter, and that exposure to a combination of such temperature cycles and those of mechanical agitation resulted in the 'correct' semilunar synchronisation.

The range of entrainment of the circadian component was examined by exposing cultures of *C. marinus* to light cycles (LD) whose periods (T) were close to 24 hours and considered to span the primary range of entrainment (Neumann and Heimbach, 1985; Neumann, 1989). Strong entrainment of semilunar timing was only possible in semimonthly *Zeitgeber* cycles combined with light cycle periods between LD 11:11 (T = 22 hours) and LD 14:14 (T = 28 hours). Semimonthly peaks of eclosion were much less clearly marked in shorter light cycles (LD 10:10; T = 20 hours or LD 9:9; T = 18 hours), or in longer cycles (LD 15:15; T = 30 hours). This limited range of entrainment strongly supported the hypothesis that a circadian clock was an intrinsic component of semilunar *Zeitgeber* perception.

The essential (and defining) clock property of temperature compensation was investigated for semilunar rhythmicity using a subtropical population of *Clunio tsushimensis* (Neumann, 1988). Semilunar rhythms of eclosion were established and entrained by artificial 'moonlight' cycles offered on four successive nights every 30 days in an otherwise LD 12:12 regime. Transfer of the cultures to LD 12:12 without 'moonlight', or to DD, resulted in free-running circa-semilunar rhythms that persisted for up to three months in mixed age populations. At 19°C the mean period (τ) was found to be 14.2 days, clearly less than the natural *Zeitgeber* period (T). At lower (14°C) and higher (24°C) temperatures – equivalent to mean seawater conditions in the natural habitat during winter and summer – τ was also very close to 14 days. The temperature coefficient (Q_{10}) for the 14 - 24°C range was very close to 1.0 (Fig. 15.10). These data amply demonstrate that the free-running period of the semilunar rhythm in *Clunio* is, like the periods of other 'circa'-rhythms, temperature compensated.

Temperate populations of *C. marinus* only emerge as adults during the summer months, suggesting that the winter is passed in a state of dormancy. Using a North Sea stock (Helgoland, 54°N), Neumann and Krüger (1985) demonstrated the occurrence of a larval diapause induced by short days and low water temperature (LD 8:16 at 7 or 10°C). Long days (LD 16:8) at 7-10°C, or short photophases at higher temperature, prevented diapause (Fig. 15.11). Diapausing larvae remained active and continued to feed. Metamorphosis, however,

stopped during the final larval instar during the early stages of imaginal disc formation. The dormant state was maintained for up to 5½ months, termination probably triggered by temperature rise during low tides and fine weather under lengthening days.

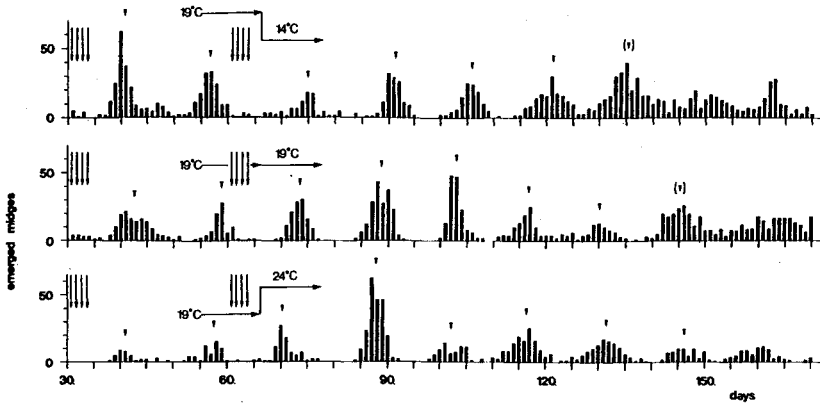


Fig. 15.10. Free-running semilunar emergence rhythms of males of *Clunio tsushimensis* at three different temperatures during LD 12:12 conditions. The lunar semi-monthly synchronisation of the three experimental groups occurred at 19°C during days 1–64 by exposure to artificial moonlight every 30 days during nights of 1–4, 31–34, and 61–64 (long arrows). Recordings of the number of midges emerging per day started on day 30 when the synchronisation became manifest. On day 66, groups 1 (above) and 3 (below) were transferred to 14° and 24°C, respectively. Arrowheads, medians of the individual emergence peaks. Arrowheads in brackets, estimate of medians with overlapping cohorts. Note the temperature compensation of the circasemilunar period. (From Neumann, 1988).

In an important and original paper, Neumann and Spindler (1991) revealed a characteristic 'switching point' in development that was controlled by the semilunar rhythm. Using synchronised populations of *C. marinus* exhibiting clear circa-semilunar rhythms of eclosion, imaginal disc development and ecdysteroid titres were followed, at 19°C, during the last (4th) larval instar. No semilunar synchronisation occurred during moulting to the fourth instar, or during the early stages of disc development. However, clear circa-semilunar 'cohorts', commencing every 15 days, became evident at about disc stage 3. Each developmental cohort reached the pupal stage after about 11 days, and eclosion followed a few days later during a well-marked semilunar 'gate'. Subsequent cohorts were triggered at 15-day intervals. Developmental times between the switching point and pupation, and the rates of disc maturation, were temperature compensated between 11 and 19°C. Ecdysteroid titres remained low during disc stages 1 to 7, increasing substantially during prepupal and pupal development. In advance of the switching point (disc stage 3), however, a small but pronounced rise in ecdysteroids was observed, perhaps suggesting a causal relationship between ecdysteroid titre and developmental switching.

Semilunar Rhythms in Other Insects

There are few other studies suggesting semilunar rhythmicity in insects, and none comparable to the *Clunio* case with its outstanding depth of treatment. Rounds (1981) prepared midday ethanol extracts of nervous tissue (brain, sub-oesophageal ganglion, and thoracic and

abdominal ganglia) of the cockroach *Periplaneta americana* and recorded peaks of neurotransmitter-like activity, particularly for the sub-oesophageal ganglion, close to full and new moon. The cockroaches were kept throughout under LD 12:12 (or in a greenhouse under semi-natural conditions) and the author made no observations which might have addressed the question of endogeneity. Interpretation of the phenomenon was that "animals were receiving pulses of gravitational maxima, and they could at least potentially be used as a timing mechanism". In a later paper, Rounds (1983) applied acetylcholine and noradrenaline daily to semi-isolated hearts of *P. americana*. The response to acetylcholine varied with a semilunar periodicity; that for noradrenaline appeared to be lunar. In both studies the author favoured an exogenous response to uncontrolled geophysical variables rather than an endogenous rhythmicity. Without further investigation this issue remains unresolved.

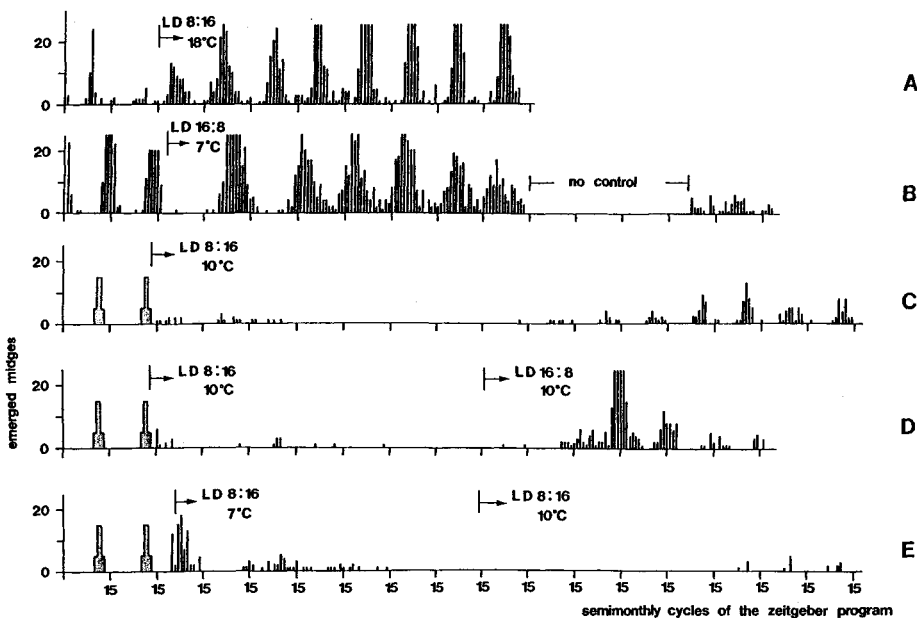


Fig. 15.11. *Clunio marinus* (Helgoland stock). Showing the influence of photoperiod and low temperature on the semilunar emergence pattern of laboratory populations. Transfer of populations to short day length and low temperature (cultures C, D and E) resulted in a pronounced delay in emergence representing a larval diapause. (From Neumann and Krüger, 1985).

Lunar Rhythms

Lunar rhythms of activity or adult emergence (with a period of about 28 days) have been described under field conditions for a number of insects (e.g. Hartland-Rowe, 1955; Corbet, 1958; Fryer, 1959; Kerfoot, 1967). Danthanarayana (1986) reviewed lunar periodicity in flight activity and migration of insects under natural conditions. Using data from various sources (light trapping, suction trapping, visual swarm counts etc) such activity was recorded in most of the major insect orders. Lunar rhythms of flight activity in honeybees (Oehmke, 1973) and of blood cardioacceleratory activity in the cockroach *Periplaneta americana* (Rounds, 1975,

1983) have also been reported. In at least two examples (Hartland-Rowe, 1958; Youthed and Moran, 1969b) these rhythms appear to be endogenous.

Hartland-Rowe (1955, 1958) showed that the mayfly *Povilla adusta* emerged from the waters of Lake Victoria in its greatest numbers just after full moon. This rhythm was maintained after the nymphs had been kept in the dark for 10 days, and in two individuals, for 6 weeks. The second case concerns the rhythm of pit-building activity by larvae of the antlion *Myrmeleon obscurus*. Using mean pit volume as a measure of activity, Youthed and Moran (1969b) showed that maximum activity occurred at the time of the full moon (Fig. 15.12). There was also a clear lunar-day (24.8 hour) rhythm with a peak in activity about 4 hours after moonrise. The authors demonstrated that the observed lunar rhythm (period about 28 days) was a combination of this lunar-day and the solar-day (circadian) rhythm described earlier (Youthed and Moran, 1969a), and which produced a peak in activity shortly after dark. This was achieved by subjecting twelve larvae of *M. obscurus* to a reversed light-cycle in which the 14-hour dark period began at 9.45 a.m. instead of at its normal time. This treatment rapidly reversed the solar-day activity pattern, but the lunar-day activity peak still occurred about 4 hours after moonrise. Results showed that the lunar rhythm reached its maximum at the new moon rather than at the full moon. The lunar rhythm of pit-building activity was also shown to 'free-run' for at least two or three cycles (92 days) in DD, but damp out in LL; it was therefore considered to be endogenous. A lunar rhythm was absent, however, in larvae reared since hatching in the absence of moonlight. The functional significance of the lunar rhythm was unclear.

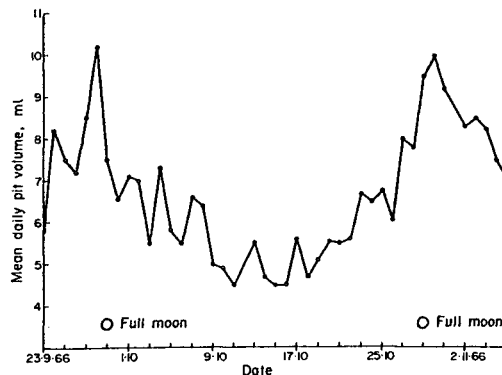


Fig. 15.12. Mean daily pit volume of a group of fifty ant lion (*Myrmeleon obscurus*) subjected to normal daylight conditions, and each larva fed one ant a day. Open circles indicate the times of the full moon. (From Youthed and Moran, 1969b.)

Tidal Rhythms

Purely tidal rhythms, with an endogenous period (τ) close to 12.4 hours, are uncommon in insects because few of them have invaded the sea or its littoral zones. A few examples, however, have been reported for insects and mites of the intertidal 'crevice' fauna. On the Pacific coast of North America, for example, the carabid beetle *Thalassotrechus barbarae* forages in the open at night and at low tide, but retreats to crevices during the day and at high tide (Evans, 1976). Nocturnal locomotor activity is under the control of a circadian rhythm ($\tau = 23.9$ hours), stable in constant conditions for up to seven cycles, and an endogenous circatidal

rhythm ($\tau = 12.4$ hours) which persists for about three cycles. The circatidal rhythm appears to be 'subordinate' to the circadian rhythm, but serves to inhibit locomotor activity during periods of nocturnal high water. The saltmarsh carabid *Dicheirotrichus gustavi*, on the other hand, appears to possess no endogenous tidal or semilunar components, nocturnal circadian activity becoming suppressed after two or more consecutive tidal inundations by apparently exogenous means (Treherne and Foster, 1977).

Foster and Moreton (1981) showed that the collembolan *Anurida maritima* had a well-defined tidal rhythm in its marine saltmarsh habitat. A large proportion of the insects emerged from underground refuges shortly after retreat of the tide and then burrowed underground again about one hour before its return. In the field this rhythm followed the tides with a strong 12.4 hour periodicity and persisted during sequences of non-submerging tides. Surface activity, however, was suppressed when low tides occurred at night. In the laboratory, away from tides and under constant light, the activity rhythm persisted for at least four days with a period (τ) of about 12.3 hours, shorter than the environmental tidal cycle. The rhythmic activity of *A. maritima*, which clearly functions to enable the insects to anticipate tidal return, was therefore regulated by an endogenous circatidal rhythm, modified by interaction with probably exogenous nocturnal suppression of activity.

Among the mites of the intertidal fauna, tidal rhythms have been described in *Ameronothrus marinus* (Schulte, 1976) and *Bdella interrupta* (Treherne et al., 1977). The former lives on rocky shores of the North and Baltic seas, where it feeds on algae. The North Sea population shows rhythms of locomotion, feeding, and defaecation, all with an endogenous period of about 12.3 hours; they persist in the absence of tidal stimulation, and free-run in daily light-cycles. In the tideless Baltic, however, activity is synchronised in daily cycles with no tidal component. The predatory mite *Bdella interrupta* occurs on marine salt marshes exposed to regular tidal inundation during the high spring tides, but not during the neaps. There are two peaks of activity per day. During the spring or submerging tides these peaks occur every 12.5 hours (or twice every 25 hours), apparently entrained to the tidal cycle. During the neap or non-submerging tides, however, the activity cycles free-run with a period of about 11.5 hours (or twice in 22.9 hours). The rhythm is therefore insensitive to the daily light-cycle as an entraining agent, and indicates true circatidal rhythmicity.

In a more recent paper, Meyer-Rochow and Brown (1998) studied the activity patterns of the beetle *Chaerodes trachyscelides* on a New Zealand beach. In its natural environment this beetle is confined to beach debris of washed-up seaweed which moves up and down with the tide. In the laboratory, locomotor activity was studied under LD 12:12 for 6 days and then under continuous darkness for a further month. Under LD 12:12 activity was strictly nocturnal. In darkness the actogram showed periodic 'scalloping' in both starts and finishes of activity, apparently caused by an interaction with an additional, tidal element. The authors interpreted the apparent 7-day cycle of advancing and delaying bands of activity as an example of a 'circa-septan' (7 day) rhythm. However, an alternative hypothesis could be that an element of the endogenous circatidal component acted as a *Zeitgeber* for the circadian oscillator. When one of the twice-daily tidal components (the phase marking high tide?) fell during late subjective night (SN) or early subjective day (SD) - and the other tidal high at a phase where it caused a smaller phase shift - the result would be a phase advance ($+\Delta\phi$) in circadian activity. Conversely, when one of the tidal components fell in the late SD or early SN - and the other at a phase causing little or no phase shift - the net result would be phase delay ($-\Delta\phi$). Periodic advances and delays in the onset of locomotor activity could therefore be regulated by interactions between endogenous circadian and circatidal oscillators and it becomes unnecessary to invoke a 'circa-septan' rhythm.

D. CIRCANNUAL RHYTHMS AND 'LONG-RANGE' TIMERS

The use of day length as an indicator of season is widespread in the insects and other organisms, the 'information' so gained being used to synchronise a variety of developmental and physiological phenomena to the appropriate season (Chapter 9). Apart from the winter and summer solstice, however, each day length occurs twice in a year, once when photophases are increasing in the spring and once when they are decreasing in the autumn. Although responses to the *direction* of such changes are known (Chapter 10), many insects are not 'required' to make such a differentiation. This may be because (1) the developmental stages sensitive to day length may only be present at the appropriate season (i.e. autumn), or (2) cold spring weather, especially at higher latitudes, may delay the resumption of activity well beyond the critical day length. Nevertheless many long-lived animals such as birds, mammals and reptiles have to distinguish between spring and autumn and the direction of seasonal change: one way this is achieved is by utilising an endogenous 'calendar' based on a biological oscillation with a period close to a year (i.e. a *circannual* rhythm).

Circannual rhythms governing seasonal cycles of migration, moulting and breeding in vertebrates have been known for several decades. Such rhythms occur in birds (Gwinner, 1967, 1971), mammals (Pengelley and Fisher, 1963; Goss, 1969 a, b; Heller and Poulson, 1970) and lizards (Stebbins, 1963). Circannual rhythms are also known for the cave crayfish *Orconectes pellucidus* (Jegla and Poulson, 1970). Gwinner (1971) found annual cycles of *Zugunruhe* (migratory restlessness), moulting and body weight in warblers, which persisted in an unchanging photoperiodic regime (LD 12:12) for several cycles. In the absence of a natural *Zeitgeber* (which is thought by Goss, 1969a, b, to be the annual changes in day length) these endogenous cycles deviated considerably from their entrained period of 12 months. In starlings, for example, the free-running period for the circannual rhythm of testicular size was about 9½ months (Schwab, 1971).

Circannual rhythms governing seasonal cycles are also to be found in some long-lived insects. Indeed, the best-documented example apparently antedates any of those mentioned above but seems to have escaped the attention it deserves. In a series of experiments, Blake (1958, 1959) described an endogenous circannual rhythm controlling diapause and the rate of development of the 'carpet' beetle *Anthrenus verbasci*. The range of this work equals any of the later work with birds and mammals and provides some remarkable parallels to the circadian rhythm controlling eclosion in fruit flies (Chapter 3).

In their natural habitat the larvae of *A. verbasci* feed on material of animal origin and are commonly found in house sparrows' nests. The life cycle generally occupies two years (semi-voltine) with the first winter spent in diapause as a young larva and the second as a full-grown larva, again in diapause. Some individuals may take the three or more years to complete their development. After the second or last diapause the larvae pupate and the adults emerge the following spring. Blake (1958) showed that when the larvae were reared in the laboratory in constant conditions of temperature and humidity, and in continuous darkness (or at least in a dark incubator opened periodically to enable pupae to be collected), this rhythm of diapause and development persisted, thereby demonstrating its endogeneity. In the absence of its natural *Zeitgeber*, which was subsequently shown to be changing day length (see below), the period of the rhythm was found to be between 41 and 44 weeks, rather than the 52 weeks found in the entrained condition. Blake (1958) recognised this endogenous 41-week interval as the rhythm's 'basic periodicity'.

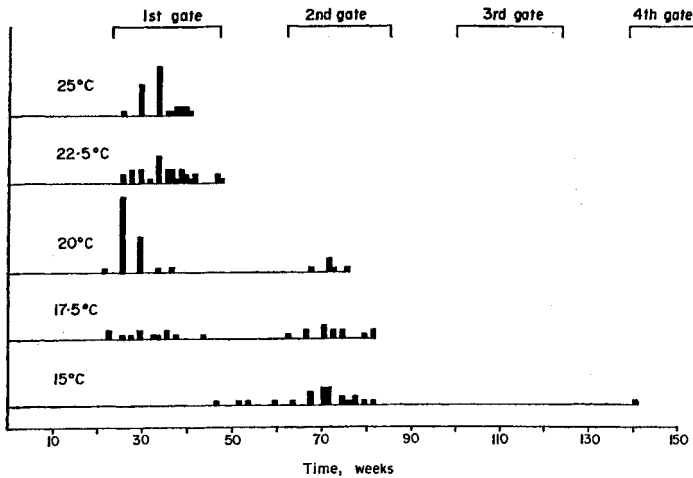


Fig. 15.13. The circannual rhythm of pupation in the carpet beetle, *Anthrenus verbasci*. Frequency of pupation times when larval development has occurred in constant conditions of temperature, humidity and darkness. A black square represents the time of pupation, to the nearest week, of an individual. Note that the larvae at higher temperature are able to utilise the first gate; at lower temperature an increasing proportion of them are required to wait until the next. (From Blake, 1959. Published by the courtesy of the Pest Infestation Control Laboratory, Ministry of Agriculture, Fisheries, and Food, Slough, England. Crown Copyright is reserved by the Controller, Britannic Majesty's Stationery Office.)

The period of the rhythm in the laboratory remained comparatively unaffected by either stationary photoperiods or by temperature (i.e. it was temperature-compensated). Populations of larvae maintained in DD and in different constant temperatures differed, however, in the proportion utilising each pupation peak (Blake, 1958, 1959). Thus at high temperature (25° and 22.5°C) development was rapid and all larvae pupated in the first peak, whereas at low temperature (15°C) all the larvae underwent two cycles of development and diapause, and pupated in the second peak about 41 weeks after the first. Depending on the temperature, therefore, the insects were either univoltine or semivoltine. Larvae maintained at intermediate temperatures (20° and 17.5°C), however, were 'split', some utilising the first and some the second pupation peak (Fig. 15.13). This remarkable experiment clearly demonstrated that the circannual rhythm of pupation in *Anthrenus verbasci* was a 'gated' phenomenon comparable to the similar gated control of pupal eclosion in *Drosophila pseudoobscura* (Pittendrigh, 1966; Skopik and Pittendrigh, 1967), but on an annual rather than a daily time scale. In other words, at the intermediate temperatures, any larva 'missing' the first gate had to wait a full cycle of 41 weeks before it could pupate.

In subsequent papers, Blake (1960, 1963) showed that the *Zeitgeber* entraining the circannual rhythm in *A. verbasci* to an exact yearly period was the natural seasonal change in day length. Both increasing and decreasing day lengths were shown to influence the rate of development. Blake (1960) maintained four groups of larvae at different combinations of outdoor temperature, constant temperature (20°C), naturally changing day lengths, and constant darkness. Under conditions of continuous darkness and constant temperature (Fig. 15.14D) the endogenous rhythm free-ran and the second peak occurred, as expected, about 40

to 44 weeks after the first. Under conditions of constant temperature and natural day length (C), however, the second peak of pupation was delayed by about 13 weeks. It appeared that the naturally decreasing day length experienced by the larvae had delayed pupation from October to January, thereby lengthening the second cycle to about 52 weeks. In biological terms it ensured that subsequent pupal development and eclosion were restricted to the following spring, and represented the entrainment of the free-running circannual rhythm ($\tau \sim 41$ weeks) to the environmental year ($T = 52$ weeks). Under outdoor conditions of temperature, populations of larvae maintained in natural day length, or in DD (A and B), pupated at the same time, suggesting that light played no part in synchronisation. These results, therefore, were also a strong indication that the natural seasonal change in temperature acted as an important *Zeitgeber*.

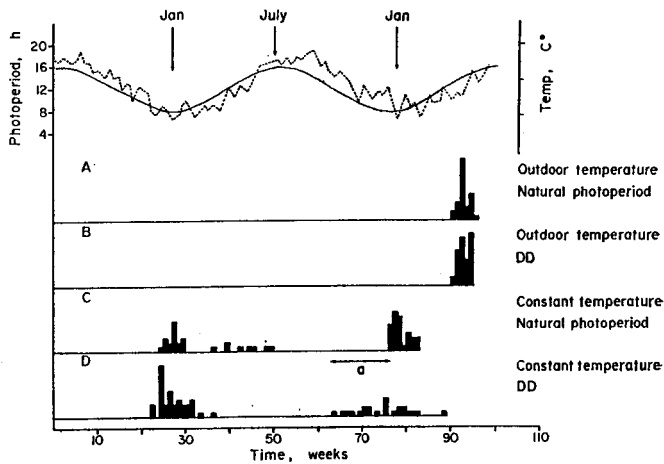


Fig. 15.14. Frequency of pupation times when larval development of *Anthrenus verbasci* has taken place under various combinations of light and temperature. C and D, constant temperature (20 °C). One black square marks the time of pupation, to the nearest week, of one individual. Observations were made weekly. A - the delay in pupation (13 weeks) caused by the exposure of the larvae to decreasing photoperiod. (From Blake, 1963.)

It was later demonstrated that the first cycle, as illustrated by the development of univoltine individuals, was controlled in a different way from the second (Blake, 1963) (Fig. 15.15). During the first cycle the length of the larval period was *decreased* whenever larvae were reared in increasing photophases during early larval life, whereas the second cycle was delayed by decreasing photophases so that most individuals pupated in January and February. Advance and delay phase-shifts are clearly recognisable in these phenomena.

This important phenomenon has been recently (and emphatically) confirmed by Numata and Nisimura (2001) using Japanese populations of *A. verbasci*. Rearing larvae under unchanging short days (LD 12:12) and a range of constant temperatures (17.5, 20, 22.5, 25 and 27.5°C) some larvae (gate 1) were found to pupate 25 to 30 weeks after hatching, but most pupated about 40 weeks later, equivalent to gate 2. In one series at 20°C, a third pupation group (gate 3) occurred about 40 weeks after the second. An endogenous circannual rhythm in *A. verbasci* was thus confirmed. To address the problem of the *Zeitgeber* involved, larvae were transferred at different ages from stationary long days (LD 16:8) to short days (LD 12:12), or

vice versa, at a constant temperature of 20°C. In most cases, pupation occurred about 23 weeks after transfer; the change in photoperiod from long to short probably entrained the circannual rhythm. Rearing larvae at 20°C under different stationary photoperiods (LD 13:11, LD 14:10, LD 15:9 and LD 16:8) showed that LD 13:11 was similar in effect to LD 12:12, but under the longer photophases only a small proportion of the larvae showed later, sporadic, pupation. There was evidence, therefore, for a critical day length between 13 and 14 hours per day.

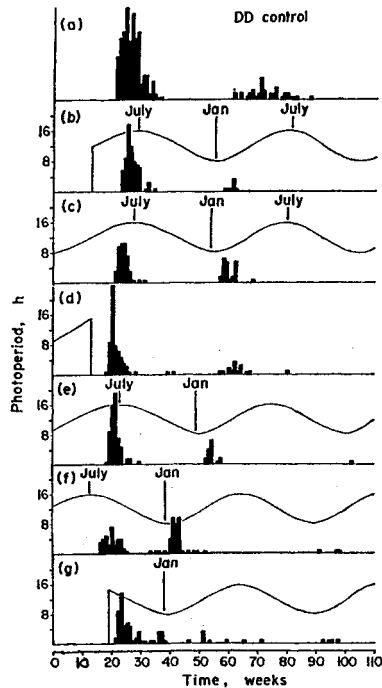


Fig. 15.15. Frequency distribution of pupation times in *Anthrenus verbasci* when larval development has taken place in various combinations of natural day length and DD at 20 °C, 70 per cent r.h. Experiments (c), (d), (e) and (f) commenced during the months of increasing day length; note that the developmental period of the univoltine insects (1st peak) is shortened. The sine wave indicates the natural changes in day length, the area below the sine-wave the length of the photoperiod. One black square marks the time of pupation (to the nearest week) of one individual. (From Blake, 1963. Published by the courtesy of the Pest Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, Slough, England. Crown Copyright is reserved by the Controller, Her Britannic Majesty's Stationery Office.)

An apparent circannual rhythmicity has also been reported for the photoperiodic response of the spider mite *Tetranychus urticae* (Razumova, 1978). Mites were maintained for three years in conditions of constant temperature (20°C) and constant short days (LD 12:12) and found to have maximum diapause in the winter (80 to 90 per cent) but minimum (down to 0 per cent) in the summer. No mention was made of the period of this circannual rhythm, or whether it was free-running in LD 12:12, but it was reported to be relatively independent of temperature between 15 and 25°C. A putative annual rhythm of sensitivity to insecticide was

also found for the house fly *Musca domestica* maintained under apparently constant conditions (Reinhardt, 1978).

Short-lived insects such as aphids can also distinguish spring from autumn but by means of an apparently non-rhythmic 'interval timer' which may extend its effects over several generations, thereby preventing the aphids from responding prematurely to short days in the spring (Bonnemaïson, 1951; Lees, 1960b; Dixon, 1971, 1972). In the green vetch aphid *Megoura viciae*, for example, the over-wintering diapause egg gives rise to a specialised virginopara (the fundatrix) which gives birth to successive generations of viviparous and parthenogenetic offspring. Lees (1960b) found that if such a clone was exposed continuously to a short photophase (at 15°C) no sexuales (males and oviparae) were produced for at least 90 days, a time covering several generations of virginoparae. After this period, the photoperiodic response was suddenly restored and oviparae were produced under the short-day treatment.

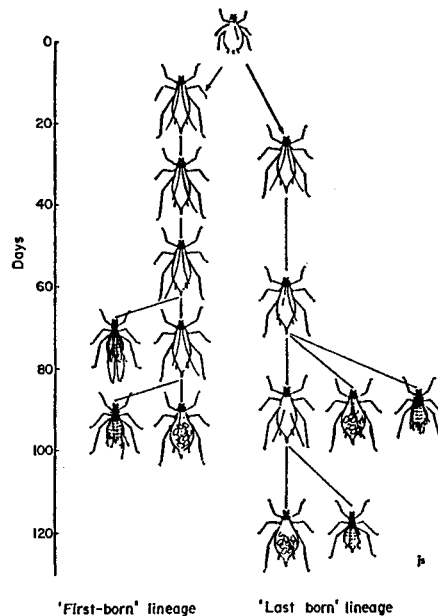


Fig. 15.16. A trans-generation interval timer in the aphid *Megoura viciae*. Two lineages, started from a single fertilised egg and a single fundatrix (above) were exposed permanently to a short-day regime. The appearance of males (winged) and oviparae is delayed for 75 to 90 days, irrespective of the generation. (From Lees, 1970.)

Working with *M. viciae*, Lees (1960) bred from either the first-born progeny in each generation, or from the last-born, the generation time of the latter being almost doubled by this procedure. Results showed that oviparae were produced on almost the same day in both lineages even though the first-born line had been through seven generations and the last-born line merely four (Fig. 15.16). Thus the long-range 'interval timer' was dependent on the passage of time rather than the number of generations. The timer, however, showed a marked temperature-dependence. At 20°C oviparae appeared in the first-born lineage after 32 to 44 days, at 15°C they appeared after 55 to 76 days, and at 11°C after 114 days. The timer also appeared to be independent of the hourglass-like clock thought to be responsible for night

length measurement in this insect. In young clones maintained under long day length (LD 16:8) the capacity to produce oviparae remained latent, but became apparent after transfer to short days provided that the timer had run its course.

E. ULTRADIAN COURTSHIP RHYTHMS

In males of *Drosophila* spp a courtship 'song', produced by vibration of the wings, consists of pulses of tone produced at intervals of about 34 msec. Intervals between successive pulse trains fluctuate rhythmically, with a period of about a minute in *D. melanogaster* and 0.5 minutes in *D. simulans*. In a study of such *ultradian* (short period) rhythms, Kyriacou and Hall (1980) found that interpeak intervals (IPIs) were affected by mutation at the *period* locus (see Chapter 4) known in *D. melanogaster* for its period-altering effect on circadian rhythms of locomotion and pupal eclosion (Konopka and Benzer, 1971). In flies with a Canton-S background, Kyriacou and Hall showed that wild type (*per*⁺) flies displayed IPIs of about 56 seconds, whereas IPIs for the short period mutant (*per*^S) were about 40 seconds and those for the long period mutant (*per*^L) about 76 seconds. *per*^O males, apparently arrhythmic on the circadian time-scale, also seemed to reveal no systematic sinusoidal pattern in their courtship song. These important observations thus revealed an extraordinary parallel effect of the same gene on rhythmic parameters of quite different periods.

Kyriacou and Hall (1980) raised wild type (Canton-S) males of *D. melanogaster* at 29°C and transferred them to either 16° or 35°C to record their song cycles. Although data sets were small and rather incomplete, IPIs at 16°C were found to be about 53 seconds, whereas those at 35°C were about 51 seconds, indicating a high degree of temperature compensation. In view of the importance of temperature compensation (see Chapter 16) it is surprising that further and more complete analyses of this nature have not been performed.

The original observations of Kyriacou and Hall were met by a series of claims and counterclaims for their very existence. This controversy is outside the scope of the present chapter, but left the subject in need of independent investigation and more 'sensitive' forms of data analysis. This was provided by Alt et al. (1998) using more sophisticated spectral methods such as correlogram and maximum entropy spectral analysis (MESA). The new investigation revealed mean IPIs of 67.95 seconds for *per*⁺, 45.9 seconds for *per*^S and 72.05 seconds for *per*^L. 'Arrhythmic' flies (*per*^O) were not arrhythmic on the ultradian scale, showing IPIs of about 31.1 seconds. In the authors' opinion it was "reasonable to accept both its existence and the role of *per* in controlling it".

A biological function for the song cycle was investigated by Ritchie et al. (1999) who played synthetic recordings with peak interval parameters typical of *D. melanogaster*, *D. simulans* and *D. sechellia* to *D. melanogaster* females in the presence of 'mute' males. It was found that females mated most rapidly when stimulated by song typical of their own species. Between *Drosophila* spp., therefore, courtship song may influence sexual isolation.

The four classical 'circa-rhythms' (circadian, circatidal, circalunar and circannual; Aschoff, 1981) show endogenous periods close to an appropriate environmental variable (day, tide, month or year), sensitivity to an environmental *Zeitgeber* to effect entrainment, and a high degree of temperature compensation of period (τ) without which entrainment could not occur. These properties enable circa-rhythms to 'time' behavioural and physiological events in relation to predictable environmental change, thereby matching internal organisation to external cycles. In the case of ultradian rhythms such as the song cycle, period ($\tau \sim$ a minute) has no obvious relationship to the environment, and mechanisms for entrainment are lacking (and indeed not required). The period of the courtship rhythm, however, appears to be

temperature compensated: the period itself has biological significance, being buffered against adventitious temperature change. The rhythm thus acts as a 'biological clock' in that *time* (period) is significant, not merely its rhythmicity.

ANNOTATED SUMMARY

1. The bees' *Zeitgedächtnis* (time memory) is an endogenous circadian clock that enables them to 'remember' the time of the day at which nectar sources are available. The oscillator free-runs in LL with a natural period (τ) slightly less than 24 hours, and shows many similarities to the overt behavioural rhythms described in Chapter 2. It differs, however, in that any phase of the oscillation can be used as a time reference (i.e. it is 'continuously consulted'), and two or more such 'training times' can be established per day provided they are separated by intervals greater than two hours.
2. Bees and some other insects can orientate themselves to the sun, to the pattern of polarised light from the sky, or to an artificial light source. A constant compass direction is maintained because the orientational mechanism also contains a circadian clock that compensates for the sun's apparent movement during the day.
3. Rhythms with a tidal periodicity are obviously rare in insects, but the marine Chironomid *Clunio marinus* shows a semilunar rhythm of emergence from the pupa that restricts eclosion to a 2-hour period at the lowest spring low waters, occurring roughly twice a month (every 14.7 days). This periodicity is a 'combination' of a circadian rhythm controlling eclosion itself, and a semilunar rhythm determining the beginning of pupation. Both components may free-run in constant laboratory conditions. Variation between geographical strains of *C. marinus* and other marine Chironomids are discussed. Some temperate populations of *C. marinus* overwinter in a photoperiodically regulated larval diapause.
4. Lunar rhythms with a period close to a month have been described in a few insects such as the mayfly *Povilla adusta* that emerges from the waters of Lake Victoria just after full moon.
5. Purely tidal rhythms, with a period close to 12.4 hours, are known to occur in a few beetles, mites and a collembolan living in intertidal habitats. Tidal rhythmicity may interact with a circadian oscillator to modify (suppress or enhance) activity.
6. Circannual rhythms with a natural period (τ) close to a year have been described with certainty only in the 'carpet' beetle *Anthrenus verbasci*. This rhythm controls pupation in this essentially semi-voltine species, although all the larvae will pupate in the first year's 'gate' in higher temperatures. In constant conditions the oscillation free-runs with a period (τ) between 41 and 44 weeks, but is entrained to a strictly annual period by the seasonal changes in photoperiod.
7. Aphids, such as *Megoura viciae*, may possess a non-oscillatory and 'transgeneration' timer that serves to prevent them from producing premature oviparae during the long nights of spring. After an interval of several weeks, which is not temperature compensated, the ability to respond to photoperiod is suddenly restored.
8. Ultradian rhythms, with endogenous periods shorter than the circadian range, are known in a number of insects (see also Chapter 6). In *Drosophila melanogaster* and other *Drosophila* spp. such rhythms regulate a male courtship 'song', produced by wing vibration. This rhythm has a period of about a minute, is temperature compensated, and is influenced by mutation at the *period* locus. It seems to serve a function in sexual isolation between species.

CHAPTER 16

CLOCK COMPLEXITY: THE WAY FORWARD?

Everything should be as simple as possible - but not simpler!
Albert Einstein

CONTENTS

Introduction	473
A. <i>Complexity in the Environment: multiple Zeitgeber and input pathways</i>	474
B. <i>'Clock' Complexity: what is a biological 'clock', anyway?</i>	476
C. <i>Temperature Compensation: a hard nut to crack</i>	476
D. <i>Pacemakers and Slaves: an outstanding problem</i>	478
E. <i>Photoperiodic Time Measurement: a prime function of the circadian system</i>	479
F. <i>Non-circadian Oscillations: the problem of very short and very long periods</i>	481

INTRODUCTION

WE are frequently urged to apply Occam's razor to elaborate data sets – that is, to explain them in the simplest terms consistent with reality. In order to make rapid scientific progress we should of course continue to pursue the parsimonious aspects of this fourteenth century precept but, at the same time, not lose sight of the undoubted complexity of biological organisation.

The complexity of biological organisation, as applied to chronobiology, was consistently stressed in the writings of C.S. Pittendrigh. For more than 40 years he pointed out that circadian rhythmicity was a *cellular* phenomenon (Pittendrigh, 1958, 1960, 1993) so that complex organisms (such as insects) were populations of interacting (cellular) oscillators. Nevertheless, progress was made by treating animals (and even populations of animals) as 'single' oscillators. This minimalist approach clarified the oscillatory properties of such rhythmic systems and facilitated determination of the principles of entrainment (Chapters 2 and 3). The simple input (entrainment) → clock (oscillator) → output (overt rhythm) model (Chapter 8) also led to the identification of some of the major physiological components of the regulatory pathways involved. However, during recent decades we have learned more of the complexity of the circadian system: nested loops of biochemical oscillators, positive and negative feedback, cellular and physiological interactions, clocks with different functions in different tissues and organs etc.

In insects, as in other organisms, complexity exists at all levels of organisation; many of these complexities have been hardly touched experimentally. There are complexities in the environmental signals (*Zeitgeber*) used by insects as entraining agents. There are complexities

arising from the multicellular organisation of the circadian system, in the input-output pathways, and how current molecular models explain the canonical features of a 'clock', particularly temperature compensation. There are unexplained complexities arising from hierarchical arrangements of pacemakers and slaves. And, above all, the role of circadian oscillations in photoperiodic induction is still unexplained, as is the mode of regulation of other 'circa' rhythms with tidal, lunar and annual periodicity. This final chapter will briefly examine some of these problems and complexities, sometimes with reference to work on taxa other than the insects. The unravelling of these problems will surely come to dominate chronobiology as we enter the 21st century.

A. COMPLEXITY IN THE ENVIRONMENT

As soon as Dawn with her rose-tinted hands had hit the East. Homer

In insects, as in other organisms, there are multiple environmental entraining agents (*Zeitgeber*). For circadian rhythms, by far the most important is the daily cycle of light and darkness generated by the revolution of the earth on its axis. Also important is the daily cycle of temperature (thermoperiod) that is correlated with the light cycle, days being warmer than nights. For tidal, semilunar, lunar and circannual rhythms there is a variety of entraining agents including cycles of tidal inundation and exposure, nocturnal illumination by the moon, and annual changes in day length. Ample evidence for the importance of these *Zeitgeber* has been presented in this book. Multiple and disparate *Zeitgeber* suggest multiple and disparate input pathways to the relevant driving oscillator(s). Even for photic entrainment of the circadian clock there may be multiple inputs to the driving oscillator, even within the same species. Thus circadian photoreceptors may include compound eyes, extra-retinal photoreceptors within the central nervous system, and direct photic input (via cryptochrome; see Chapter 4) to the biochemical feedback loop constituting the pacemaker.

The daily cycle of light is in itself, of course, a complex signal. With regard to its action as a *Zeitgeber* for the entrainment of circadian rhythms, it changes through the day in both irradiance and spectral quality. The 'height' of the sun above the horizon (its azimuth) also varies with the time of day (Roenneberg and Foster, 1997). Most organisms use the *twilight* transitions at dawn and dusk as indicators of the phase of the day, and in photoperiodism it is frequently the *dawn* transition that operates the physiological 'switch' between the nondiapauses and diapause pathways (Chapter 10). It is important therefore to study irradiance and spectral quality of the light at these times. With rather few exceptions, notably those concerned with action spectra (Chapters 2, 3 and 10), the majority of insect investigations have used simple, square-wave cycles of white light and darkness with the intensity of light above some known minimum threshold. Natural light intensity (irradiance), however, may increase by six or seven orders of magnitude between the first glimmers of dawn and noon on a cloudless summer's day (see Fig. 2.14 for an insect example). In addition, spectral quality at the important twilight times may be comparatively enriched by shorter wavelengths (Roenneberg and Foster, 1997). Irradiance may also vary over the day with cloud cover or changes in the behaviour of the animal, giving rise to substantial 'noise' in the photic signal which may challenge the entrainment mechanism.

Among vertebrates, so-called 'social' *Zeitgeber* may also be important. Examples for circadian rhythms include bird song cycles (Gwinner, 1966) or daily feeding schedules (Hau and Gwinner, 1992) which may serve to entrain behavioural cycles. Examples of such

Zeitgeber seem to be uncommon in the insect literature, although a notable exception seems to occur in honey bee foraging (see Chapter 2).

Some entrainment phenomena appear at first sight to be components of the 'output' pathway. In hamsters, two hour bouts of 'novelty-induced' running activity (in a newly introduced running wheel, for example) were found to entrain the locomotor activity rhythm (Mrosovsky and Salmon, 1987; Mrosovsky et al., 1989). The phase response curve for such activity pulses was found to be a mirror image to that for light: phase advances ($+\Delta\phi$) between Ct 4 and 14, and delays ($-\Delta\phi$) between Ct 14 and 2. Activity-induced, 'non-photic', *Zeitgeber* have not apparently been recorded for insects, but similar phenomena have been described for the scorpion *Androctonus australis* (Fleissner and Fleissner, 1992). In the blow fly *Calliphora vicina*, however, topical application of an ecdysteroid agonist caused phase shifts of the locomotor rhythm and a phase response curve which was a 'mirror image' of that for light (Cymborowski et al., 1993). This may indicate that natural endogenous pulses of ecdysteroids 'feedback' through the 'output' pathway to the central behavioural oscillator.

On the *input* pathway to the central oscillator are circadian events in the eyes and optic lobes (see also Chapter 5). In the house fly *Musca domestica* rhythms of anatomical and physiological change occur in the compound eyes that are regulated by a circadian pacemaker in the optic lobe lamina, a pacemaker location distinct from the midbrain site for the behavioural pacemaker in flies (probably the lateral neurons; see Chapters 4 and 8). In the house fly these rhythms effect changes in the photoreceptor terminals which persist with a circadian frequency in DD (Pyza and Meinertzhagen, 1993; Meinertzhagen and Pyza, 1996). The significance of these input pathway rhythms is probably that they cause the photic input to the brain to be also rhythmic. This may in turn help the central behavioural oscillator to cope with the 'noisy' photic input, allowing active 'probing' for a signal and to modulate the strength of a given physical stimulation (light) according to circadian time. In this respect it is of interest to recall (Chapter 2 and 3) that light pulses have their greatest phase shifting action at times when light is normally absent, during the subjective night. Roenneberg and Mellow (1998, 2001) and Lakin-Thomas (2000) have called the actively probing process a *Zeitnehmer* (time taker) (Fig. 16.1). Such *Zeitnehmer* loops may possess other important functions in the complex circadian system, such as regulation of period length and robustness of the rhythm, which are only just being appreciated.

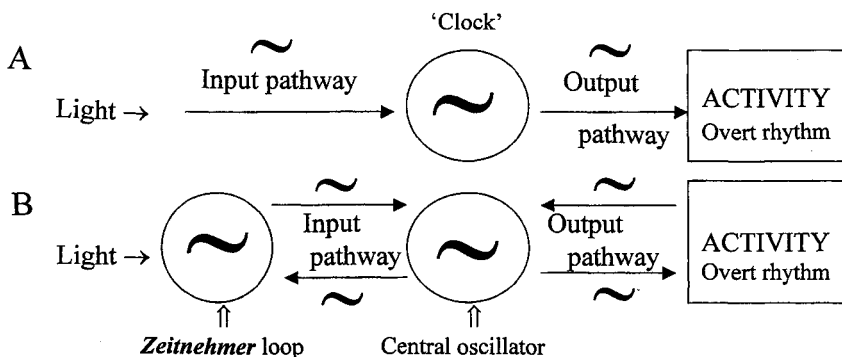


Fig. 16.1. Models for the circadian system. A – a simple input→pacemaker→output model. B – a more likely model incorporating a rhythmic *Zeitnehmer* loop on the input pathway, and feedback from the output to the central pacemaker (after Roenneberg and Mellow).

B. 'CLOCK' COMPLEXITY

In general it is a good morning exercise for a researcher to destroy a favourite hypothesis every day before breakfast – it keeps him young. Konrad Lorenz

Circadian systems in insects, as in other complex organisms, are themselves complex. This is because circadian phenomena are essentially cellular phenomena and complex animals are composed of many cells. As outlined in earlier chapters, insect circadian 'pacemakers' that regulate behavioural or physiological rhythms may be found at all levels of organisation from tissues through organs to whole animals. Roenneberg and Merrow (1998) outlined the 'canonical' features of such systems. These include *rhythmicity as such*, independent of its circadian frequency; the *circadian range*; whether the rhythm is *sufficiently self-sustained*; *temperature compensation* of the circadian period; and the *entrainability* of such rhythms to external *Zeitgeber*. They asked whether all of these functions may be explained by the expression of just a few genes within a molecular feedback loop (Chapter 4), or whether they might be generated by cellular interactions or by circadian feedbacks in the input or output pathways. In such systems cells, tissues and organs must possess mutually coupled oscillatory processes, 'communicating' to each other by means of neural, hormonal or signal transduction pathways. This raises a question about the smallest component that may be regarded as a *bona fide* clock. Does the cell possess all circadian functions from input to output, or do groups of cells making up a central oscillator rely on rhythmic photic inputs from other systems (eyes for example), on cell-cell coupling for period regulation, or on rhythmic output pathways for their expression? In the first case the cell may be regarded as a (complete) circadian clock; in the latter case the clock is a multicellular, even multi-organ structure perhaps relying on the whole animal for its expression. Functional clocks almost certainly lie at all levels of organisation, and the circadian system is undoubtedly much more complex than that envisaged a few years ago.

At the cellular level there are complications existing or emerging with regard to the current *per-tim* feedback model for the circadian clock (see Chapter 4). These include whether the genes so far identified are 'dedicated' clock genes or whether they have additional and more general functions and have merely been hi-jacked into a feedback loop to serve a circadian function. In some insects, and in some tissues (brain neurosecretory cells of *Antheraea pernyi*, for example; Sauman and Reppert, 1996a; Rosato and Kyriacou, 2001) PER and TIM proteins do not seem to enter the nucleus, an essential feature of the current loop model. Even in *D. melanogaster* a functional *per* gene may not be essential for all circadian phenomena. The 'morning' locomotor activity peak may persist, for example, in *per*⁰ flies (Helfrich-Forster, 2000), the visual sensitivity rhythm has been reported to be unaffected by mutation of this gene (Chen et al., 1992), and photoperiodic time measurement may proceed in this gene's absence (Saunders et al., 1989). Perhaps there are other genes with a 'clock' function, as yet undiscovered? The existence of such genes should be actively pursued.

C. TEMPERATURE COMPENSATION

In the early 1950s when Colin Pittendrigh met Albert Einstein in Princeton, Pittendrigh's account of his work on Drosophila eclosion rhythms apparently cut little ice until Pitt mentioned temperature compensation. "Now you're talking!" said Einstein.

It has long been recognised that the 'natural' period of a free-running circadian oscillation (τ) is homeostatically buffered against temperature, and that this feature of circadian (and other 'circa' rhythms) is one of their vital characteristics. In his seminal paper on the eclosion rhythm of *Drosophila pseudoobscura*, discussed at greater length in Chapter 3, Pittendrigh (1954) showed that intervals between eclosion peaks were almost the same between 16° and 26°C after certain temperature-induced transients had subsided. The relationship between temperature and τ revealed a temperature coefficient (Q_{10}) characteristically close to unity (in this case, 1.02). In a review given at the Cold Spring Harbor Symposium on Biological Clocks, Sweeney and Hastings (1960) showed that such temperature compensation was perhaps universal, occurring across all taxa from unicellulars to mammals. Pittendrigh stressed that although the circadian *period* (τ) was so regulated, other aspects of the eclosion phenomenon such as 'amplitude' (number of flies emerging within the daily gate) or the phase relationship (ψ) between eclosion peak and light on, were not (see Chapter 3). It was only circadian *period* that was so compensated, strongly suggesting its evolutionary significance. Throughout his life Pittendrigh (e.g. Pittendrigh, 1993) stressed that the temperature compensation of τ was a "functional prerequisite" for time measurement. This was true not only in a trivial sense (how can a clock tell the time if it is affected by temperature?), but more importantly because *entrainment* to external *Zeitgeber* would fail if τ lengthened or shortened much beyond a period close to 24 hours.

Although the central importance of temperature compensation has been recognised for over half a century, satisfactory explanations for the phenomenon are most notable by their absence. An early idea (e.g. Sweeney and Hastings, 1960) was that the circadian output was based on the mutual coupling of two temperature-dependent oscillators with complementary temperature coefficients. In such an appropriately coupled physico-chemical mechanism, the rate of one of the processes could be decreasing with temperature, and the other rising, such that their *net* coupled output became constant. With this model, 'over-compensation' (see Chapters 2 and 3) could arise because of inaccuracy in either 'direction'.

When the *period* mutants of *D. melanogaster* were isolated in the early 1970s (see Chapter 4) the short-period mutant (*per^S*; τ about 19 hours) and the long-period mutant (*per^L*; τ about 29 hours) were found to have an 'opposite' dependence of τ on temperature (Konopka et al., 1989; see Fig. 2.8). Pittendrigh played with the idea that the two mutants represented complementary oscillators coming together in wild type flies to provide a τ value close to 24 hours over a wide range of temperatures. By the 1990s (Pittendrigh, 1993) these 'magic numbers' (i.e. $19 + 29 = 48/2 = 24$ hours) had been rejected on the grounds that both mutations mapped to the same exon (see Chapter 4); the general principle of opposing components, however, probably remains as valid as ever. In formal feedback models for circadian rhythms (see Chapter 7) the rate of increase of the postulated and essential chemical was thought to be greater at higher temperature. On the other hand, the temperature insensitive, passive loss or degradation phase began at a higher concentration so taking longer to decline; hence the overall circadian period remained comparatively unchanged.

At the molecular level antagonistic processes were again invoked for this phenomenon (Huang et al., 1995). In *D. melanogaster* these authors suggested that the overall temperature compensation of τ might in part be due to temperature independent PER protein activity based on competition between antagonistic interactions each with a similar temperature coefficient.

A different basis for temperature compensation was proposed by Sawyer et al. (1997) from work on natural variation in the *period* gene from populations of *D. melanogaster* isolated from a wide north-south range in Europe and North Africa. It was demonstrated that the threonine-glycine (Thre-Gly) repeat region of PER was markedly polymorphic in length.

The major variants Thre-Gly 17 and 20 were distributed in a highly significant latitudinal cline with the former being more abundant at southern latitudes and the latter predominating in northern Europe. This distribution was apparently related to the flies' ability to maintain circadian period at different temperatures. It was suggested that the cline may have evolved as a function of temperature, although it was not clear which *aspect* of the seasonally variable temperature pattern might be important or, indeed, if other variables (such as photoperiod?) were involved. Nevertheless, it was proposed that the more robust temperature compensation of the Thre-Gly 20 allele might be at a premium in colder and more thermally variable environments.

It is clear from the above account that the nature and mode of generation of temperature compensation is an unresolved issue. Satisfactory explanation of this vital circadian property remains one of the outstanding problems.

D. PACEMAKERS AND SLAVES

Any feedback loop in the organism is a potential slave oscillator, and if the circadian pacemaker can make input to the loop, the slaves will become part of the temporal program that the pacemaker drives. C. S. Pittendrigh (1981)

Chapter 6 stressed the multi-oscillatory nature of the insect circadian system, not only in the trivial sense that insects are multicellular animals, but in the more profound sense that circadian outputs are frequently the product of multicellular driving systems depending for at least some of their properties on cell-cell interactions. One of the oldest and most persuasive examples of this is the hierarchical pacemaker-slave structure proposed for pupal eclosion in *Drosophila pseudoobscura*. Extensive analysis of this two-tier system (see Chapter 6) convinced C.S. Pittendrigh of its widespread occurrence and led to the quotation that heads this section.

In its original form, dating from 1957 (Pittendrigh and Bruce, 1957) a central self-sustained, temperature compensated and light entrainable 'pacemaker' was thought to drive unilaterally a downstream 'slave' that was perhaps less temperature compensated, not light entrainable and perhaps also damping. It was the pacemaker that received temporal information from the environment but the slave that controlled eclosion. A persuasive body of data accumulated to support this two-oscillator model, but eclosion rhythmicity in *D. pseudoobscura* remained the only well-documented case. Physiological and molecular analyses of circadian systems have since identified components on the output pathway from 'central' oscillators but the pacemaker-slave model (specifically for eclosion) has not been investigated at this level.

Jackson et al. (2001) have reviewed components of the circadian output pathway (see also Chapter 4). In different species, candidates include pigment dispersing factor (PDF), the prothoracicotrophic hormone (PTTH)–ecdysteroid axis, and the eclosion hormone (EH) system. These might present some of the properties of a 'slave' system. In *D. melanogaster*, the *lark* gene affects aspects of pupal eclosion but not locomotor activity, and is therefore part of the output pathway to eclosion rather than a component of the central oscillator (Newby and Jackson, 1993, 1996). LARK protein shows a circadian cycle, but *lark* mRNA shows no rhythmic changes in abundance; the LARK cycle must therefore depend on a post-transcriptional mechanism. Since *lark* is not expressed circadianly it is not considered to be a 'slave' oscillator although, of course, it could be oscillatory but heavily damped without being driven by an upstream and self-sustained pacemaker.

Wider acceptance of the pacemaker-slave model has probably been hampered by its over-prescriptive nature. Hierarchical chains of events are commonplace in biochemistry and physiology. In the present context, the brain-prothoracic gland axis controlling moulting through the agency of the hormonal cascade from PTTH to ecdysteroids is a good example. Perhaps the PTTH 'clock' in the brain is a pacemaker, and the ecdysteroid clock in the prothoracic glands is a slave? Work by Steel and Vafopoulou (Chapter 5) demonstrates that circadian 'pulses' of PTTH from the brain of *Rhodnius prolixus* entrain the rhythm of ecdysteroid synthesis and release from the prothoracic glands, thereby suggesting a vertical pacemaker-slave system. In this case, however, the prothoracic gland contains a self-sustaining, temperature compensated and light entrainable circadian clock that is capable of fulfilling these functions in the absence of the brain. Is it therefore a slave or a pacemaker? At this point it is probably best to recognise that the original two-oscillator model is entirely appropriate for *Drosophila* eclosion but is otherwise too prescriptive. Vertical coupling probably abounds in insect rhythms but some 'driving' oscillations may show damping, and some 'driven' systems may be independently entrained by the light; the terms 'pacemaker' and 'slave' should therefore be used with caution. The undoubtedly widespread occurrence of linked oscillators, however, gives credence to the 'pacemaker-slave' concept, and future investigations should certainly address this problem more directly.

E. PHOTOPERIODIC TIME MEASUREMENT

*The human mind treats a new idea like the way the body treats a strange protein
– it rejects it.*

Sir Peter Medawar

Photoperiodism is undoubtedly one of the most important functions of the circadian system. However, when compared to overt behavioural rhythms, progress in this field has remained comparatively slow, with molecular analyses notable by their absence. This is partly due to the complexity of the photoperiodic response that, unlike locomotor rhythmicity for example, encompasses a large part of the insect's developmental programme. The complex concatenation often spans (1) photoperiodic sensitivity during an earlier instar, (2) measurement of night length by the 'clock', (3) accumulation of successive nights (long or short) by the 'counter', and (4) ultimate expression at the diapausing stage. This may span several metamorphic events, or even from one generation to another (Chapter 10). During this time known (e.g. successive endocrine events) and unknown (e.g. time measurement and 'counting') processes are occurring. Classical mutational analysis would therefore be difficult: mutations affecting diapause might be occurring at any level of this long cascade, from photoreception to endocrine regulation.

What do we know about photoperiodic time measurement itself? We know (or think we know) that night length measurement is a function of the circadian system (Chapter 11) which, however, may be severely damped to resemble a dark period 'hourglass'. In many cases this system appears to be independent of the circadian oscillations regulating overt rhythmicity, and may indeed be independent of known molecular feedback loops.

The critical day length for photoperiodic diapause induction in *D. melanogaster* appears to be identical in both the short period (*per^S*) and long period (*per^L*) mutations (Saunders, 1990), thereby suggesting, perhaps, that the *period* gene is not causally involved in photoperiodic time measurement. However, τ for the locomotor rhythm in *per^L* is shortened towards wild type values as the temperature is lowered (to 15°C), but that for *per^S* is lengthened (Konopka et al., 1989; Fig. 2.8). It is thus conceivable that important phase

relationships between the oscillators and light may be almost identical at the rather low temperature (12°C) required for diapause induction, thereby making this observation less compelling. However, discrimination between long and short days also proceeds in overtly arrhythmic *per*⁰ mutant flies or even in the complete absence of the *per* gene (Saunders et al., 1989), albeit with a shortened critical day length. This again suggests that photoperiodic time measurement may be independent of a 'central' oscillator incorporating *per* transcription as part of its circadian machinery. In this context, there is some evidence for *per*-independent rhythmicity in *D. melanogaster*. Adult locomotor activity is bimodal, with morning (M) and evening (E) peaks (Helfrich-Förster, 2001; see also Chapters 2 and 4). The phase relationship of E to the light cycle is affected by mutation at *per* (Figure 2.11) and its entrainment to light is through the agency of cryptochrome. The morning (M) activity peak, on the other hand, is apparently unaffected by *per* mutation and its entrainment appears to be through the compound eyes. *per*⁰ flies may also retain the morning peak which is regulated by a short-period (τ 20 – 22 hours) oscillation that dampens quite rapidly in DD.

Helfrich-Förster (2001) has noted similarities between the M oscillator and the putative photoperiodic oscillator that regulates ovarian diapause (weak persistence in DD) and has postulated that they may be identical, the short period of M leading to a short critical day length in *per*⁰ flies. However, although this proposition contains some attractive features, it cannot be the case. A short value of τ would lead to all circadian phases phase-leading the light cycle. This in turn would lead to a short critical night length – or a *long* critical day length – quite the opposite of what is observed in photoperiodic experiments. Abundant evidence also suggests that the photic input for photoperiodism in flies is direct to the brain (rather than, or in addition to, a pathway through the compound eyes).

Recent molecular screening in *D. melanogaster* using DNA microarrays (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Chapter 4) has uncovered a large number of genes (other than known clock genes) whose mRNAs exhibit circadian oscillations in their concentration. Some of these will undoubtedly prove to be driven elements on the output pathways of known clock genes, but others could be independent pacemakers or rhythmic 'slaves'. Any of them could be involved in phenomena such as photoperiodic timing. Clearly we should look elsewhere for possible *per*-independent photoperiodic oscillators.

The very large number of studies on insect photoperiodism has given rise to a large number of formal clock models (reviewed in Chapter 13). If these are examined on a chronological basis they show a progression from simple to more complex models, but with an increasing plausibility because of increased understanding of the system within the context of known circadian properties. There is little doubt that Bünning's *general* hypothesis is correct in some form or another. However, some *specific* models such as 'External coincidence' and 'Internal coincidence' that looked so promising 30 years ago have not fulfilled that promise. Unequivocal tests have not been devised to verify or reject their major propositions, or to distinguish between them. Our current understanding of photoperiodic time measurement includes elements of both types of model. Progress in the analysis of this important area of chronobiology urgently requires answers to the following questions: How many oscillators are involved, and what is the molecular nature of their feedback loops? Is the photoinducible phase a physiological reality? And what is the nature of the process by which long or short nights accumulate during the sensitive period?

In a constructive but frankly speculative essay on the possible molecular basis for PPTM, Tauber and Kyriacou (2001) have turned their attention to other known 'clock' genes in *D. melanogaster*. In particular, they focus on the possible roles of *timeless* and *cryptochrome*, which could act independently of *period*.

Although *D. melanogaster* with its unrivalled genetic background has provided a foundation for uncovering the molecular basis of the circadian mechanism in both locomotor and eclosion rhythmicity (see Chapter 4), it is probably less useful as a model for photoperiodism. Further studies should examine species with a much more robust response (at higher temperature), a short 'chain of command' between light perception and diapause regulation, a well-studied background in diapause induction, and the possibility to study photoperiodism and overt circadian behaviour concurrently. Such a species may be found among other drosophilids - such as *D. auraria* or *D. littoralis* - that live at higher latitudes and over-winter with a robust photoperiodic diapause. Using such a system, progress might then be made using modern techniques to disrupt canonical circadian homologues (such as dsRNAi technology) to reveal what, if any, role those clock components play in photoperiodic timing.

At least three scenarios arising from this approach are possible: (1) Disruption of known 'clock' genes eliminates overt rhythmicity (locomotor and eclosion), photoperiodic timing (PPTM) and Nanda-Hamner (N-H) periodicity. (2) Gene disruption eliminates overt rhythmicity, but leaves N-H periodicity and PPTM running as usual. Or (3) such disruption eliminates overt rhythmicity and N-H periodicity, but leaves photoperiodic timing untouched. The first possibility might indicate that all three phenomena are functions of the known '*per-tim*' loop; the second that Nanda-Hamner periodicity is an expression of the photoperiodic oscillator which is separate (distinct from) such a loop; and the third that Nanda-Hamner periodicity is an expression of *basic* circadian rhythmicity, but PPTM is a separate mechanism. Such experimental approaches might help us to discriminate between several of the more important models reviewed in Chapter 13. In all three scenarios, however, PPTM might conceivably be a 'novel' time measuring mechanism (such as an 'hourglass'), or - as is preferred in this book - the outcome of a *strongly damped* ('hourglass-like') circadian oscillator.

One thing is sure: analysis of photoperiodism will be a more difficult problem than that of overt rhythmicity, and it presents a considerable future challenge.

F. NON-CIRCADIAN OSCILLATIONS

You ain't seen nuthin' yet. Ronald Reagan.

Endogenous clock-like oscillators ('*circa-rhythms*') with a wide range of periods have been the subject matter of this book. They range from short period *ultradian* oscillations (period τ from a few seconds or minutes up to about 20 hours), through *circatidal* (τ about 12 to 13 hours), *circadian* (τ 19 to 30 hours), *circasemilunar* (τ 14 to 15 days) and *circalunar* (τ about 29 days) to *circannual* rhythms (τ about a year). Apart from some of the ultradian rhythms, they show the common properties of a period close to a natural cycle, temperature compensation (of τ) and an ability to synchronise (entrain) their activity to a cyclical variable (tide, day, month or year) in the natural environment. In so doing they achieve a characteristic *phase relationship* (ψ) to the environmental cycle which allows them to perform behavioural and physiological 'events' at a time that is clearly of selective advantage and great evolutionary significance. In short, clock-like oscillations are a reflection *within the organism* of its cyclical environment, allowing it to perform 'at the right time', frequently anticipating environmental change and preparing for it rather than merely responding to it with a maladaptive knee-jerk. Most information, by far, is available for circadian rhythms including

molecular models suggesting how the circadian period is generated (Chapter 4). The generation and regulation of other rhythmic systems, however, is practically unknown.

Longer period ultradian, circatidal and circadian oscillators (τ values ranging from about 12 to 30 hours) may possibly be explained on the basis of proposed molecular feedback loops or similar interactive cycles (Chapter 4). They would differ in the time delays within the loop (to generate different periods) and in the nature of the *Zeitgeber* (tide or light for example) acting *via* the input pathways. However, very much shorter ultradian cycles, in the order of minutes or seconds, probably have a cycle time far too short for conventional transcription-translation loops involving passage of mRNAs and proteins from nucleus to cytoplasm and *vice versa*. The role of *per* in the courtship rhythm of *D. melanogaster*, for example, must have a different basis to that proposed for circadian rhythms.

How then are the longer periods of circasemilunar, circalunar and circannual rhythms generated? Work reviewed in Chapter 15 shows that each of these systems possesses a temperature compensated free-running period (τ) that approximates the relevant environmental period (tide, phases of the moon, or year) and entrains to those environmental periods under both natural and laboratory conditions. Pulses of the relevant *Zeitgeber* stimulus also elicit a characteristic phase response curve with the same period. Does this mean that longer period oscillations are governed by endogenous cycles of that length? Molecular feedback loops lasting for a month (or a year) are indeed difficult to imagine.

In taxa other than the insects some authors have attempted to attribute longer period oscillations to interaction between two shorter oscillations according to the 'beat principle'. For example, the semilunar rhythm of spore discharge in the brown alga *Dictyota dichotoma* may be the result of an interaction between a circadian rhythm and a circatidal rhythm (Bünning and Müller, 1962). Such an interaction was indicated in experiments where the algae were exposed to light cycles differing from 24 hours, spore discharge occurring every 11 to 12 days under LD 13.5:9.5 ($T = 23$ hours) but every 16 to 17 days under LD 14.25:10.25 ($T = 24.5$ hours).

Very few experiments of this type have been conducted using insect material. Some evidence in favour of the 'beat' hypothesis was obtained for the lunar rhythm of pit-building activity by larvae of the ant lion (Youthed and Moran, 1969; Chapter 15), but extensive work on semilunar rhythmicity in *Clunio marinus* (Neumann, 1976b; Chapter 15) led to its rejection. How non-circadian periodicity is generated thus remains an important question for the future.

Postscript and prospect: It has been the intention of this book to present modern aspects of insect chronobiology within an historical framework. In this way current advances in insect physiology and molecular biology, for example, are linked with the pioneering work of the 'giants' of chronobiology such as Jürgen Aschoff, Stanley Beck, Erwin Bünning, Alexander Danilevskii, Tony Lees, Colin Pittendrigh and Carroll Williams - all now sadly deceased, but not forgotten. Such a linkage ensures that today's investigators do not lose sight of the foundations of the subject. It is considered essential that modern experimenters keep an eye on the older literature which so often provides the *raison d'être* for more modern studies. In particular, 'classical' investigations that established the canonical features of insect clocks should form the bases of current molecular approaches. These must include, not just how the pacemaker generates its rhythmicity, but also the attendant and vital properties of circadian (or circatidal, circalunar or circannual) period, its temperature compensation and its entrainment by the appropriate environmental *Zeitgeber*. More specifically, the complex and characteristic responses of circadian systems to, *inter alia*, 'skeleton' photoperiods, light and temperature pulses of different 'strengths' given at different phases, and the complex responses of insects to

seasonal changes in day length must all be explained. 'Classical' experimental designs - such as the T-experiment, the bistability phenomenon, phase response curves, 'stopping' the clock by accurately timed pulses of light or extended light periods - should provide the framework for sophisticated investigations unravelling the true nature of insect clocks.

This Page Intentionally Left Blank

REFERENCES

A man will turn over half a library to make one book. Samuel Johnson

- ADAMS, A.J. (1986a) Night-interruption experiments and action spectra for dawn and dusk in relation to the photoperiodic clock of the cabbage whitefly, *Aleyrodes proletella* (Homoptera: Aleyrodidae). *J. Insect Physiol.* **32**, 71-78.
- ADAMS, A.J. (1986b) The photoperiodic clock in the cabbage whitefly, *Aleyrodes proletella*: Resonance experiments at three temperatures. *J. Insect Physiol.* **32**, 567-572.
- ADAMS, M.E. and ZITMAN, D. (1997) Identification of ecdysis-triggering hormone in the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Comm.* **230**, 188-191.
- ADEDOKUN, T.A. and DENLINGER, D.L. (1984) Cold hardiness: a component of the diapause syndrome in pupae of the flesh flies, *Sarcophaga crassipalpis* and *S. bullata*. *Physiol. Entomol.* **9**, 361-364.
- ADKISSON, P. L. (1961) Effect of larval diet on the seasonal occurrence of diapause in the pink bollworm. *J. econ. Ent.* **54**, 1107-1112.
- ADKISSON, P. L. (1964) Action of the photoperiod in controlling insect diapause. *Am. Nat.* **98**, 357-374.
- ADKISSON, P. L. (1966) Internal clocks and insect diapause. *Science, Wash.* **154**, 234-241.
- ADKISSON, P. L., BELL, R. A. and WELLSO, S. G. (1963) Environmental factors controlling the induction of diapause in the pink bollworm, *Pectinophora gossypiella* (Saunders). *J. Insect Physiol.* **9**, 299-31
- ADKISSON, P. L. and ROACH, S. H. (1971) A mechanism for seasonal discrimination in the photoperiodic induction of pupal diapause in the bollworm *Heliothis zea* (Boddie). In *Biochronometry* (Ed. MENAKER, M.), pp. 272-280. National Academy of Sciences, Washington.
- AGUI, N. (1975) Activation of prothoracic glands by brains *in vitro*. *J. Insect Physiol.* **21**, 903-913.
- AIDA, S. (1963) *Seibutsu Kagaku*, **15**, 163-167. (Quoted from Sakai and Masaki, 1965.) (In Japanese.)
- AIDA, S. and SAKAGAMI, Y. (1962) *Kagaku*, **32**, 96-97. (in Japanese) (Quoted from Sakai and Masaki, 1965.)
- ALLADA, R., WHITE, N.E., SO, W.V., HALL, J.C. and ROSBASH, M. (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell* **93**, 791-804.
- ALLEMAND, R. (1974) Importance evolutive du comportement de ponte chez les insectes: comparaison du rythme circadien d'oviposition chez les six especes de *Drosophila* du sous-groupe *melanogaster*. *C. R. Acad. Sci. Paris, D*, **279**, 2075-2077.
- ALLEMAND, R. (1976a) Les rythmes de vitellogenese et d'ovulation en photoperiode LD 12:12 de *Drosophila melanogaster*. *J. Insect Physiol.* **22**, 1031-1035.
- ALLEMAND, R. (1976b) Influence de modifications des conditions lumineuses sur les rythmes circadiens de vitellogenese et d'ovulation chez *Drosophila melanogaster*. *J. Insect Physiol.* **22**, 1075-1080.
- ALLEMAND, R. (1976c) Importance adaptative du rythme circadien de ponte chez les drosophilides: comparaison de huit especes du genre *Zaprionus*. *C. R. Acad. Sci. Paris, D*, **282**, 85-88.
- ALLEMAND, R. (1977) Influence de l'intensite d'eclaircissement sur l'expression du rythme journalier d'oviposition de *Drosophila melanogaster* en conditions lumineuses LD 12:12. *C. R. Acad. Sci. Paris, D*, **284**, 1553-1556.
- ALLEMAND, R. (1983) The circadian oviposition rhythm of *Drosophila melanogaster*: 2. Influence of biotic factors. *Biol. of Behav.* **8**, 273-288.
- ALLEN, G., RAPPE, J., EARNEST, D.J. and CASSONE, V.M. (2001) Oscillating on borrowed time: Diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. *J. Neuroscience* **21**, 7937-7943.
- ALSTEIN, M., GAZIT, Y., BEN AZIZ, O., GABAY, T., MARKUS, R., VOGEL, Z. and BARG, J. (1996) Induction of cuticular elanization in *Spodoptera littoralis* larvae by PBAN/MRCH: Development of a quantitative bioassay and structure function analysis. *Arch. Insect Biochem. Physiol.* **31**, 355-370.
- ALT, S., RINGO, J., TALYN, B., BRAY, W. and DOWSE, H. (1998) The *period* gene controls courtship song cycles in *Drosophila melanogaster*. *Animal Behaviour* **56**, 87-97.
- AMPLEFORD, E.J. and DAVEY, K.G. (1989) Egg laying in the insect *Rhodnius prolixus* is timed in a circadian fashion. *J. Insect Physiol.* **35**, 183-187.
- AMPLEFORD, E.J. and STEEL, C.G.H. (1982) Circadian control of ecdysis in *Rhodnius prolixus* (Hemiptera). *J. Comp. Physiol. A* **147**, 281-286.
- AMPLEFORD, E.J. and STEEL, C.G.H. (1985) Circadian control of a daily rhythm in haemolymph ecdysteroid

- titre in the insect *Rhodnius prolixus* (Hemiptera). *Gen. comp. Endocrin.* **59**, 453-459.
- AMPLEFORD, E.J. and STEEL, C.G.H. (1986) Induction of rhythmic modulation of haemolymph ecdysteroids in the insect *Rhodnius prolixus* by treatment which elicit rhythmic ecdysis. *Gen. Comp. Endocrinol.* **63**, 353-361.
- ANDERSON, J. F. (1968) Influence of photoperiod and temperature on the induction of diapause in *Aedes atropalpus* (Diptera: Culicidae). *Entomologia exp. appl.* **11**, 321-330.
- ANDO, Y. (1978) Studies on egg diapause in the false melon beetle, *Atrachya menetriesi* Faldermann (Coleoptera: Chrysomelidae). *Bull. Fac. Agric. Hirosaki Univ.* **30**, 131-215 [In Japanese, English Summary].
- ANDRETIC, R., CHANEY, S. and HIRSH, J. (1999) Requirement of circadian genes for cocaine sensitization in *Drosophila*. *Science* **285**, 1066-1068.
- ANKERSMIT, G. W. (1968) The photoperiod as a control agent against *Adoxophyes reticulana* (Lepidoptera: Tortricidae). *Entomologia exp. appl.* **11**, 231-240.
- ANKERSMIT, G. W. and ADKISSON, P. W. (1967) Photoperiodic responses of certain geographical strains of *Pectinophora gossypiella* (Lepidoptera). *J. Insect Physiol.* **13**, 553-564.
- ANTON, S. and GADENNE, C. (1999) Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. *Proc. Natl. Acad. Sci., USA* **96**, 5764-5767.
- ARAI, T. (1975) Diel activity rhythms in the life history of the oriental fruit fly, *Dacus dorsalis* Hendel (Diptera: Trypetidae). *Jap. J. appl. Ent. Zool.* **19**, 253-259. (In Japanese.)
- ARAI, T. (1976a) Effects of temperature and light-dark cycles on the diel rhythm of emergence in the oriental fruit fly, *Dacus dorsalis* Hendel (Diptera: Trypetidae). *Jap. J. appl. Ent. Zool.* **20**, 69-76. (In Japanese.)
- ARAI, T. (1976b) Effects of light and temperature on the diel cyclicity in the larval jumping behavior of the oriental fruit fly, *Dacus dorsalis* Hendel (Diptera: Trypetidae). *Jap. J. appl. Ent. Zool.* **20**, 9-14 (In Japanese.)
- ARAI, T. (1977) Effects of the daily cycle of light and temperature on hatchability and hatching time in *Metrioptera hime* Furukawa (Orthoptera, Tettigonidae). *Kontyu*, **45**, 107-120.
- ARAI, T. (1998a) Effects of non-24 h photoperiods on the hatching time in *Metrioptera hime* (Orthoptera: Tettigonidae). *Entomol. Sci.* **1**, 1-6.
- ARAI, T. (1998b) Effect of photoperiod and thermoperiod on hatching rhythm in *Homorocoryphus jezoensis* Matsumura et Shiraki (Orthoptera: Tettigonidae). *Entomol. Sci.* **1**, 491-494.
- ARAI, T. (1998c) Egg hatching rhythms of *Eobiana engelhardti subtropica* Bey-Bienko (Orthoptera: Tettigonidae). *Entomol. Sci.* **1**, 495-501.
- ARTHUR, J. M. and HARVILL, E. K. (1937) Plant growth under continuous illumination from sodium vapor lamps supplemented by mercury arc lamps. *Contrib. Boyce Thompson Inst.* **8**, 433-443.
- ASAHINA, M., FUGO, H. and TAKEDA, S. (1994) Ecdysteroid synthesis in dissociated cells of the prothoracic gland of the silkworm, *Bombyx mori*. *Zool. Sci.* **11**, 107-111.
- ASCHOFF, J. (1960) Exogenous and endogenous components in circadian rhythms. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 11-28.
- ASCHOFF, J. (1965) Response curves in circadian periodicity. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 95-111. North-Holland, Amsterdam.
- ASCHOFF, J. (1969) Desynchronization and resynchronization of human circadian rhythms. *Aerosp. Med.* **40**, 844-849.
- ASCHOFF, J. (1981) A survey on biological rhythms. In *Handbook of Behavioral Neurobiology*, vol 4, *Biological Rhythms*. Ed J. Aschoff, Plenum Press, New York. Pp. 3-10.
- ASCHOFF, J., GERECKE, U., KURECK, A., POHL, H., RIEGER, P., v. SAINT-PAUL U. and WEVER, R. (1971) Interdependent parameters of circadian activity rhythms in birds and man, In *Biochronometry* (Ed. MENAKER, M.), pp. 3-29. National Academy of Sciences, Washington.
- ASCHOFF, J., KLOTTER, K. and WEVER, R. (1965) Circadian vocabulary. In *Circadian Clocks* (Ed. ASCHOFF, J.). North-Holland, Amsterdam.
- ASCHOFF, J. and SAINT-PAUL, U. (1982) Circadian rhythms in the blowfly, *Phormia terraenovae*: the period in constant light. *Physiol. Entomol.* **7**, 365-370.
- ASCHOFF, J. and SAINT-PAUL, U. (1990) Circadian rhythms in the blowfly, *Phormia terraenovae*: control of phase within the range of entrainment. *Physiol. Entomol.* **15**, 129-135.
- ASCHOFF, J., SAINT PAUL, U. and WEVER, R. (1971) Die Lebensdauer von Fliegen unter dem Einfluss von Zeit-verschiebungen. *Naturwiss.* **58**, 574.
- ATKINS, G. and STOUT, J. (1994) Processing of song signals in the cricket and its hormonal control. *Amer. Zool.* **34**, 655-669.

- ATWAL, A. S. (1955) Influence of temperature, photoperiod, and food on the speed of development, longevity, fecundity, and other qualities of the diamond-back moth *Plutella maculipennis* (Curtis) (Tineidae, Lepidoptera). *Aust. J. Zool.* **3**, 185-221.
- BABILIS, N.A. and MAZOMENOS, B.E. (1992) Pheromone production in *Sesamia nonagrioides*: Diel periodicity and effect of age and mating. *J. Insect Physiol.* **38**, 561-564.
- BAE, K., LEE, C., HARDIN, P.E. and EDERY, I. (2000) dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. *J. Neurosci.* **20**, 1746-1753.
- BAE, K., LEE, C., SIDOTE, D., CHANG, K.-Y. and EDERY, I. (1998) Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. *Molec. Cell. Biol.* **18**, 6142-6151.
- BAEHRECKE, E.H. (1996) Ecdysone signaling cascade and regulation of *Drosophila* metamorphosis. *Arch. Insect Biochem. Physiol.* **33**, 231-244.
- BAGNOLI, P., BRUNELLI, M., MAGNI, F. and MUSUMECI, D. (1976) Neural mechanisms underlying spontaneous flashing and its modulation in the firefly *Luciola lusitanica*. *J. Comp. Physiol.* **108**, 133-156.
- BAKER, J.D., McNABB, S.L. and TRUMAN, J.W. (1999) The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3037-3048.
- BAKER, F. C. (1935) The effect of photoperiodism on resting, treehole, mosquito larvae. *Can. Ent.* **67**, 149-153.
- BAKER, J.D., McNABB, S.L. and TRUMAN, J.W. (1999) The hormonal coordination of behaviour and physiology at adult ecdysis in *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3037-3048.
- BAKER, T.C. and CARDE, R.T. (1979) Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta*. *J. Insect Physiol.* **25** 943-950.
- BALE, J.S. (1987) Insect cold hardiness: freezing and supercooling – an ecological perspective. *J. Insect Physiol.* **33**, 899-908.
- BALE, J.S. (1993) Classes of insect cold hardiness. *Functional Ecol.* **7**, 751-753.
- BALKENOL, M. and WEBER, F. (1981) *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* **3**, 223-227.
- BALL, H. J. (1965) Photosensitivity in the terminal abdominal ganglion of *Periplaneta americana* (L.). *J. Insect Physiol.* **11**, 1311-1315.
- BALL, H. J. (1971) The receptor site for photic entrainment of a circadian activity rhythm in the cockroach *Periplaneta americana*. *Ann. ent. Soc. Am.* **64**, 1010-1015.
- BALL, H. J. (1972) Photic entrainment of circadian activity rhythms by direct brain illumination in the cockroach *Blaberus craniifer*. *J. Insect Physiol.* **18**, 2449-2455.
- BALL, H. J. and CHAUDHURY, M. F. B. (1973) Photic entrainment of circadian rhythms by illumination of implanted brain tissues in the cockroach *Blaberus craniifer*. *J. Insect Physiol.* **19**, 823-830.
- BARKER, R. J. (1963) Inhibition of diapause in *Pieris rapae* L. by supplementary photophases. *Experientia* **19**, 185.
- BARKER, R. J. and COHEN, C. F. (1965) Light-dark cycles and diapause induction in *Pieris rapae* (L.). *Entomologia exp. appl.* **8**, 27-32.
- BARKER, R. J., COHEN, C. F. and MAYER, A. (1964) Photoflashes: a potential new tool for control of insect populations. *Science, Wash.* **145**, 1195-1197.
- BARKER, R. J., MAYER, A. and COHEN, C. F. (1963) Photoperiod effects in *Pieris rapae*. *Ann. ent. Soc. Am.* **56**, 292-294.
- BARNARD, D. R. and MULLA, M. S. (1977) Effects of photoperiod and temperature on blood-feeding, oogenesis and fat body development in the mosquito, *Culiseta inornata*. *J. Insect Physiol.* **23**, 1261-1266.
- BARRET, R.K. and PAGE, T.L. ()
- BARRY, B. D. and ADKISSON, P. L. (1966) Certain aspects of the genetic factors involved in the control of larval diapause of the pink bollworm. *Ann. ent. Soc. Am.* **59**, 122-125.
- BATEMAN, M. A. (1955) The effect of light and temperature on the rhythm of pupal ecdysis in the Queensland fruit-fly, *Dacus (Strumeta) tryoni* (Frogg.). *Aust. J. Zool.* **3**, 22-33.
- BEBAS, P., CYMBOROWSKI, B. and GIEBULTOWICZ, J.M. (2001) Circadian rhythms of sperm release in males of the cotton leafworm, *Spodoptera littoralis*: in vivo and in vitro studies. *J. Insect Physiol.* **47**, 859-866.
- BEBAS, P., CYMBOROWSKI, B. and GIEBULTOWICZ, J.M. (2002) Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar H⁺-ATPase. *J. Exp. Biol.* **205**, 37-44.
- BEACH, R. F. (1978) The required day number and timely induction of diapause in geographic strains of the mosquito, *Aedes atropalpus*. *J. Insect Physiol.* **24**, 449-455.

- BEACH, R. F. and CRAIG, G. B. JR. (1977) Night length measurements by the circadian clock controlling diapause induction in the mosquito *Aedes atropalpus*. *J. Insect Physiol.* **23**, 865-870.
- BEAN, D. W. and BECK, S. D. (1980) The role of juvenile hormone in the larval diapause of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **26**, 579-584.
- BEAN, D. W. and BECK, S. D. (1983) Haemolymph ecdysteroid titres in diapause and nondiapause larvae of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **29**, 687-693.
- BEATTIE, T. M. (1971) Histology, histochemistry, and ultrastructure of neurosecretory cells in the optic lobes of the cockroach, *Periplaneta americana*. *J. Insect Physiol.* **17**, 1843-1855.
- BECK, S. D. (1962a) Photoperiodic induction of diapause in an insect. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **122**, 1-12.
- BECK, S. D. (1962b) Temperature effect on insects: Relation to periodism. *Proc. N. Central Branch, Ent. Soc. Am.* **17**, 18-19.
- BECK, S. D. (1963) Physiology and ecology of photoperiodism. *Bull. ent. Soc. Am.* **9**, 8-16.
- BECK, S. D. (1964) Time-measurement in insect photoperiodism. *Am. Nat.* **98**, 329-346.
- BECK, S. D. (1968) *Insect Photoperiodism*. Academic Press, New York and London.
- BECK, S. D. (1974a) Photoperiodic determination of insect development and diapause. I. Oscillators, hourglasses, and a determination model. *J. comp. Physiol.* **90**, 275-295.
- BECK, S. D. (1974b) Photoperiodic determination of insect development and diapause. II. The determination gate in a theoretical model. *J. comp. Physiol.* **90**, 297-310.
- BECK, S. D. (1975) Photoperiodic determination of insect development and diapause. III. Effects of nondiel photoperiods. *J. comp. Physiol.* **103**, 227-245.
- BECK, S. D. (1976a) Photoperiodic determination of insect development and diapause. IV. Effects of skeleton photoperiods. *J. comp. Physiol.* **105**, 267-277.
- BECK, S. D. (1976b) Photoperiodic determination of insect development and diapause. V. Diapause, circadian rhythms, and phase response curves, according to the Dual System theory. *J. comp. Physiol.* **107**, 97-111.
- BECK, S. D. (1977) Dual System theory of the biological clock: Effects of photoperiod, temperature, and thermoperiod on the determination of diapause. *J. Insect Physiol.* **23**, 1363-1372.
- BECK, S. D. (1980) *Insect Photoperiodism*, 2nd edition. Academic Press, New York and London.
- BECK, S. D. (1982) Thermoperiodic induction of larval diapause in the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **28**, 273-278.
- BECK, S. D. (1984) Effect of temperature on thermoperiodic determination of diapause. *J. Insect Physiol.* **30**, 383-386.
- BECK, S. D. (1985) Effects of thermoperiod on photoperiodic determination of larval diapause in *Ostrinia nubilalis*. *J. Insect Physiol.* **31**, 41-46.
- BECK, S. D. (1985) Dual system theory of the biological clock. *J. theoret. Biol.* **113**, 93-115.
- BECK, S. D. (1986) Effects of photoperiod and thermoperiod on growth of *Agrotis ipsilon* (Lepidoptera: Noctuidae). *Ann. Ent. Soc. Am.* **79**, 821-828.
- BECK, S. D. (1987) Thermoperiod-photoperiod interactions in the determination of diapause in *Ostrinia nubilalis*. *J. Insect Physiol.* **33**, 707-712.
- BECK, S. D. (1988a) Resonance in photoperiodic regulation of larval diapause in *Ostrinia nubilalis*. *J. Insect Physiol.* **35**, 75-79.
- BECK, S. D. (1988b) Thermoperiod and larval development of *Agrotis ipsilon* (Lepidoptera: Noctuidae). *Ann. Ent. Soc. Am.* **81**, 831-835.
- BECK, S. D. (1989) factors influencing the intensity of larval diapause in *Ostrinia nubilalis*. *J. Insect Physiol.* **35**, 75-79.
- BECK, S. D. and ALEXANDER, N. (1964) Chemically and photoperiodically induced diapause development in the European corn borer, *Ostrinia nubilalis*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **126**, 175-184.
- BECK, S. D. and APPLE, J. W. (1961) Effect of temperature and photoperiod on voltinism of geographical populations of the European corn borer, *Pyrausta nubilalis* (Hbn.). *J. econ. Ent.* **54**, 550-558.
- BECK, S. D., CLOUTIER, E. J. and MCLEOD, D. G. R. (1962) Photoperiod and insect development. *Proc. 23rd Biol. Colloq. Oregon State Univ.* 1962 pp. 43-64.
- BECK, S. D. and HANEC, W. (1960) Diapause in the European corn borer, *Pyrausta nubilalis* (Hüb.). *J. Insect Physiol.* **4**, 304-318.
- BEGON, M. (1976) Temporal variations in the reproductive condition of *Drosophila obscura* Fallen and *D. subobscura* Collin. *Oecologia, Berl.* **23**, 31-47.
- BEIER, W. (1968) Beeinflussung der inneren Uhr der Bienen durch Phasenverschiebung des Licht-Dunkel-Zeitgebers. *Z. Bienenforsch.* **9**, 356-378.
- BEIER, W. and LINDAUER, M. (1970) Der Sonnentand als Zeitgeber für die Biene. *Apidologie*, **1**, 5-28.

- BELANGER, J.H. and ORCHARD, I. (1993) The locust ovipositor opener muscle: proctolinergic central and peripheral neuromodulation in a centrally driven motor system. *J. exp. Biol.* **174**, 343-362.
- BELING, I. (1929) Über das Zeitgedächtnis der Bienen. *Z. vergl. Physiol.* **9**, 259-338.
- BELL, C.H. (1981) The influence of light cycle and circadian rhythm on oviposition in 5 pyralid moth pests of stored products. *Physiol. Entomol.* **6**, 231-240.
- BELL, R. A. and ADKISSON, P. L. (1964) Photoperiodic reversibility of diapause induction in an insect. *Science, Wash.* **144**, 1149-1151.
- BELOZEROV, V. N. (1964) Larval diapause in the tick *Ixodes ricinus* L. and its relation to external conditions. *Zool. Zh.* **43**, 1626-1637. (In Russian.)
- BAYLIES, M.K., VOSSHALL, L.B., SEHGAL, A. and YOUNG, M.W. (1992) New short period mutations of the *Drosophila* clock gene *per*. *Neuron* **9**, 575-581.
- BAYLIES, M.K., WEINER, L., VOSSHALL, L.B., SAEZ, L. and YOUNG, M.W. (1993) Genetics, molecular and cellular studies of the *per* locus and its products in *Drosophila melanogaster*. In *Molecular Genetics of Biological Rhythms*, Young, M.W., ed., Marcel Dekker, New York, pp. 123-153.
- BENNA, C., SCANNAPIECO, P., PICCIN, A., SANDRELLI, F., ZORDAN, M., ROSATO, E., KYRIACOU, C.P., VALLE, G. and COSTA, R. (2000) A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. *Curr. Biol.* **10**, R512-R513.
- BENNETT, M. F. and RENNER, M. (1963) The collecting performance of honey bees under laboratory conditions. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **125**, 416-430.
- BENSCHOTER, C. A. (1968) Diapause and development of *Heliothis zea* and *H. virescens* in controlled environments. *Ann. ent. Soc. Am.* **61**, 953-956.
- BENSON, J.A. and JACKLET, J.W. (1977) Circadian rhythms of output from neurones in the eye of *Aplysia*. IV. A model of the clock: differential sensitivity to light and low temperature pulses. *J. exp. Biol.* **70**, 195-211.
- BERDE, C. (1976) Nonmonotonic transients and some mathematical models of circadian rhythms. *J. theor. Biol.* **56**, 435-441.
- BERLINGER, M. J. and ANKERSMIT, G. W. (1976) Manipulation with the photoperiod as a method of control of *Adoxophyes orana* (Lepidoptera: Tortricidae). *Ent. exp. & appl.* **19**, 96-107.
- BINKLEY, S. (1979) A timekeeping enzyme in the pineal gland. *Scientific American*, **240** (4), 50-55.
- BIRUKOW, G. (1953) Menotaxis im polarisierten Licht bei *Geotrupes sylvaticus* Panz. *Naturwissenschaften*, **40**, 611.
- BIRUKOW, G. (1956) Lichtkompassorientierung beim Wasserläufer *Velia currens* F. am Tage und zur Nachtzeit. 1. Herbst- und Winterversuche. *Z. Tierpsychol.* **13**, 463-484.
- BIRUKOW, G. (1960) Innate types of chronometry in insect orientation. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 403-412.
- BIRUKOW, G. (1964) Aktivitäts- und Orientierungsrhythmik beim Kornkäfer (*Calandra granaria* L.). *Z. Tierpsychol.* **21**, 279-301.
- BIRUKOW, G. and BUSCH, H. E. (1957) Lichtkompassorientierung beim Wassertaucher *Velia currens* F. am Tage und zur Nachtzeit. II. Orientierungsrhythmik in Verschiedenen Lichtbedingungen. *Z. Tierpsychol.* **14**, 184-203.
- BJOSTAD, L.B., WOLF, W.A. and ROELOFS, W.L. (1987) Pheromone biosynthesis in lepidopterans: Desaturation and chain shortening. In: Blomquist, G.J. and Prestwich, G.D. (Eds.), *Pheromone Biosynthesis* Academic Press, Orlando, Florida, pp. 77-120.
- BLACKMAN, R. L. (1975) Photoperiodic determination of the male and female sexual morphs of *Myzus persicae*. *J. Insect Physiol.* **21**, 435-453.
- BLAIR, S.S. (1994) A role for the segment polarity gene *shaggy-zeste white 3* in the specification of regional identity in the developing wing of *Drosophila*. *Dev. Biol.* **162**, 229-244.
- BLAKE, G. M. (1958) Diapause and the regulation of development in *Anthrenus verbasci* (L.) (Col., Dermestidae). *Bull. Ent. Res.* **49**, 751-775.
- BLAKE, G. M. (1959) Control of diapause by an 'internal clock' in *Anthrenus verbasci* (L.) (Col., Dermestidae). *Nature, Lond.* **183**, 126-127.
- BLAKE, G. M. (1960) Decreasing photoperiod inhibiting metamorphosis in an insect. *Nature, Lond.* **188**, 168-169.
- BLAKE, G. M. (1963) Shortening of a diapause-controlled life cycle by means of increasing photoperiod. *Nature, Lond.* **198**, 462-463.

- BLANCHARDON, E., GRIMA, B., KLARSFELD, A., CHELOT, E., HARDIN, P.E., PRÉAT, T. and ROUYER, F. (2001) Defining the role of *Drosophila* lateral neurons in the control of activity and eclosion rhythms by targeted genetic ablation and PERIOD overexpression. *Eur. J. Neurosci.* **13**, 871-888.
- BLANEY, L. T. and HAMNER, K. C. (1957) Inter-relations among the effects of temperature, photoperiod, and dark period on floral initiation of *Biloxi* soybean. *Bot. Gaz.* **119**, 10-24.
- BLAU, J. and YOUNG, M.W. (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell* **99**, 661-671.
- BLOCK, G. and ROBINSON, G.E. (2001) Reversal of honey bee behavioural rhythms. *Nature* **410**, 1048.
- BLOCK, G.D., KHALSA, S.B.S., McMAHON, D.G., MICHEL, S. and GUESZ, M. (1993) Biological clock in the retina: cellular mechanisms of biological timekeeping. *Int. Rev. Cytol.* **146**, 83-144.
- BLOCK, G., TOMA, D.P. and ROBINSON, G.E. (2001) Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. *J. Biol. Rhythms* **16**, 444-456.
- BOLLENBACHER, W.E. and GRANGER, N.A. (1985) Endocrinology of the prothoracicotropic hormone. In: Kerkut, G.A. and Gilbert, L.I. (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol 7, 109-151. Pergamon Press, Oxford.
- BOLLENBACHER, W.E., GRANGER, N.A., KATAHIRA, E.J. and O'BRIEN, M.A. (1987) Developmental endocrinology of larval moulting in the tobacco hornworm, *Manduca sexta*. *J. exp. Biol.* **128**, 175-192.
- BOLLENBACHER, W.E., GRAY, R.S., MUEHLEISEN, D.P., REGAN, S.A. and WESTBROOK, A.L. (1993) The biology of the prothoracicotropic hormone peptidergic neurons in an insect. *Amer. Zool.* **33**, 316-323.
- BONNEMAISON, L. (1951) Contribution à l'étude des facteurs provoquant l'apparition des formes formes ailées et sexuées chez les Aphidinae. *Annls Epiphyt.* (C) **2**, 1-380.
- BONNEMAISON, L. (1958) Facteurs d'apparition des formes sexupares ou sexuées chez le puceron cendre du pommier (*Sappaphis plantaginea* Pass.). *Annls Epiphyt.* (C) **3**, 331-355.
- BONNEMAISON, L. (1965) Action d'une photopériode de durée croissantes ou décroissantes sur l'apparition des formes sexuées de *Dysaphis plantaginea* Pass. *C. R. Acad. Sci.* **260**, 5138-5140.
- BONNEMAISON, L. (1973) Action de la photopériode sur la diapause de *Mamestra brassicae* L. (Lepidopteres, Noctuidae). *C. R. Acad. Sci. Paris D*, **277**, 213-216.
- BONNEMAISON, L. (1977) Mode d'action de la photopériode sur la diapause d'*Adoxophyes orana* F. v. R. (Lepidopteres, Tortricidae). *Z. ang. Ent.* **84**, 75-99.
- BONNEMAISON, L. (1978) Effets de l'obscurité et de la lumière sur la diapause d'*Ostrinia nubilalis* Hbn. (Lep., Pyralidae). *Z. ang. Ent.* **86**, 57-66.
- BONNEMAISON, L. and MISSONNIER, J. (1955) Influence de photopériodisme sur le déterminisme des formes estivales ou hivernales et de la diapause chez *Psylla pyri* L. (Homopteres). *C. R. Acad. Sci.* **240**, 1277-1279.
- BORTHWICK, H. A., HENDRICKS, S. B. and PARKER, M. W. (1952) The reaction controlling floral initiation. *Proc. Nat. Acad. Sci. U.S.A.* **38**, 929-934.
- BOSSE, T.C. and VEERMAN, A. (1996) Involvement of vitamin A in the photoperiodic induction of diapause in the spider mite *Tetranychus urticae* is demonstrated by rearing an albino mutant on a semi-synthetic diet with and without β -carotene or vitamin A. *Physiol. Entomol.* **21**, 188-192.
- BOTELLA, L. and MENSUA, J.L. (1987) Larval diapause induced by crowding in *Chymomyza costata* (Diptera: Drosophilidae). *Annales Entomol. Fennici* **53**, 41-47.
- BOUCHER, L. and PIERRE, D. (1988) Mating rhythm of *Caryedon serratus* (Coleoptera: Bruchidae) in laboratory and natural conditions. *Ann. Soc. Entomol. France* **24**, 151-160.
- BOULIGAND, Y. (1978a) Cholesteric order in biopolymers. A.C.S. Symposium Series **74**, 237-247.
- BOULIGAND, Y. (1978b) Liquid crystalline order in biological materials. In: Blumstein, A. (Ed.), *Liquid Crystalline Order in Polymers*. Academic press, New York, pp. 261-297.
- BOUNHIOL, J.-J. and MOULINIER, C. (1965) L'opacité crânienne et ses modifications naturelles et expérimentelles chez le ver à soie. *C. R. Acad. Sci.* **261**, 2739-2741.
- BOWEN, M.F. (1990) Post-diapause sensory responsiveness in *Culex pipiens*. *J. Insect Physiol.* **36**, 923-929.
- BOWEN, M.F., BOLLENBACHER, W.E. and GILBERT, L.I. (1984) *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *manduca sexta*. *J. exp. Biol.* **108**, 9-24.
- BOWEN, M.F., DAVIS, E.E. and HAGGART, D.A. (1988) A behavioural and sensory analysis of host seeking behaviour in the diapausing mosquito *Culex pipiens*. *J. Insect Physiol.* **34**, 805-813.
- BOWEN, M.F., IRISH, R., WHISENTON, L.R., GRANGER, N.A., GILBERT, L.I. and BOLLENBACHER, W.E. (1985) Endocrine events during pre-diapause and non-diapause larval-pupal development of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* **31**, 83-90.

- BOWEN, M.F., SAUNDERS, D.S., BOLLENBACHER, W.E. and GILBERT, L.I. (1984) *In vitro* reprogramming of the photoperiodic clock in an insect brain-retrocerebral complex. *Proc. Natn. Acad. Sci. USA* **81**, 5881-5884.
- BOWEN, M. F. and SKOPIK, S. D. (1976) Insect photoperiodism: The 'T-experiment' as evidence for an hour-glass mechanism. *Science, Wash.* **192**, 59-60.
- BRADSHAW, W. E. (1969a) Major environmental factors inducing the termination of larval diapause in *Chaoborus americanus* Johannsen (Diptera: Culicidae). *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **136**, 2-8.
- BRADSHAW, W. E. (1969b) Dawn and dusk differences in the photoperiodic induction of development in *Chaoborus americanus* (Diptera: Culicidae). *Am. Zool.* **9**, 234.
- BRADSHAW, W. E. (1970) Interaction of food and photoperiod in the termination of larval diapause in *Chaoborus americanus* (Diptera: Culicidae). *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **139**, 476-484.
- BRADSHAW, W. E. (1972) Photoperiodic control in the initiation of diapause by *Chaoborus americanus* (Diptera: Culicidae). *Ann. Ent. Soc. Am.* **65**, 755-756.
- BRADSHAW, W. E. (1976) Geography of photoperiodic response in a diapausing mosquito. *Nature, Lond.* **262**, 384-386.
- BRADSHAW, W. E. and HOLZAPFEL, C. M. (1977) Interaction between photoperiod, temperature, and chilling in dormant larvae of the tree-hole mosquito, *Toxorhynchites rutilus* Coq. *Biol. Bull. mar. Biol. Lab., Woods Hole*, **152**, 147-159.
- BRADY, J. (1967a) Control of the circadian rhythm of activity in the cockroach. I. The role of the corpora cardiaca, brain and stress. *J. exp. Biol.* **47**, 155-163.
- BRADY, J. (1967b) Control of the circadian rhythm of activity in the cockroach. II. The role of the subesophageal ganglion and ventral nerve cord. *J. exp. Biol.* **47**, 165-178.
- BRADY, J. (1967c) Histological observations on circadian changes in the neurosecretory cells of cockroach subesophageal ganglia. *J. Insect Physiol.* **13**, 201-213.
- BRADY, J. (1969) How are insect circadian rhythms controlled? *Nature, Lond.* **223**, 781-784.
- BRADY, J. (1970) Characteristics of spontaneous activity in tsetse flies. *Nature, Lond.* **228**, 286-287.
- BRADY, J. (1971) The search for an insect clock. In *Biochronometry* (Ed. MENAKER, M.), pp. 517-526. National Academy of Sciences, Washington.
- BRADY, J. (1972) Spontaneous, circadian components of tsetse fly activity. *J. Insect Physiol.* **18**, 471-484.
- BRADY, J. N. (1974) The physiology of insect circadian rhythms. *Adv. Insect Physiol.* **10**, 1-115.
- BRADY, J. (1975) Circadian changes in central excitability - the origin of behavioural rhythms in tsetse flies and other animals? *J. Ent. A*, **50**, 79-95.
- BRADY, J. (1982) Circadian rhythms in animal physiology. In *Biological Timekeeping* (Ed. BRADY, J.), pp. 121-142. Society for Experimental Biology Seminar Series 14, Cambridge University Press, Cambridge.
- BRADY, J. (1987) Circadian rhythms - endogenous or exogenous? *J. comp. Physiol. A* **161**, 711-714.
- BRADY, J. and CRUMP, A. J. (1978) The control of circadian activity rhythms in tsetse flies: environment or physiological clock? *Physiol. Ent.* **3**, 179-190.
- BREMER, H. (1926) Über die tageszeitliche Konstanz im Schlupftermine der Imagines einiger Insekten und ihre experimentelle Beeinflussbarkeit. *Z. wiss. Insektenbiol.* **21**, 209-216.
- BRETT, W. J. (1955) Persistent diurnal rhythmicity in *Drosophila* emergence. *Ann. ent. Soc. Am.* **48**, 119-131.
- BRETT, W.J. (1955) persistent diurnal rhythmicity in *Drosophila* emergence. *Ann. Ent. Soc. Am.* **48**, 119-131.
- BROWER, L.P. (1977) Monarch migration. *Natural History*, June/July pp. 41-53.
- BROWN, G.C., BERRYMAN, A.A. and BOGYO, T.P. (1979) Density dependent induction of diapause in the codling moth, *Laspeyresia pomonella* (Lepidoptera: Olethreutidae). *Can. Entomol.* **111**, 431-433.
- BROWN, F. A. (1960) Response to pervasive geophysical factors and the biological clock problem. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 57-71.
- BROWN, F. A. (1965) A unified theory for biological rhythms. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 231-261. North-Holland, Amsterdam.
- BROWN, F. A., FINGERMAN, M., SANDEEN, M. I. and WEBB, H. M. (1953) Persistent Diurnal and tidal rhythms of color change in the fiddler crab, *Uca pugnax*. *J. Exp. Zool.* **123**, 29-60.
- BROWN, V.K. and HODEK, I. (1983) *Diapause and Life Cycle Strategies in Insects*. Dr W. Junk, The Hague. 283 pp.
- BRUCE, V. G. (1960) Environmental entrainment of circadian rhythms. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 29-48.
- BRUCE, V. G. and MINIS, D. H. (1969) Circadian clock action spectrum in a photoperiodic moth. *Science, Wash.* **163**, 583-585.
- BRUCE, V. G. and PITTENDRIGH, C. S. (1957) Endogenous rhythm in insects and microorganisms. *Am. Nat.* **91**, 179-195.

- BRUCE, V. G. and PITTENDRIGH, C. S. (1960) An effect of heavy water on the phase and period of the circadian rhythm in *Euglena*. *J. cell. comp. Physiol.* **56**, 25-31.
- BRUN, R. (1914) *Die Raumorientierung der Ameisen und das Orientierungsproblem im Allgemeinen*, Jena.
- BRUNNARIUS, J. and DUMORTIER, B. (1984) Existence of a light-sensitive phase in the photoperiodic termination of diapause in *Pieris brassicae* L. (Insecta: Lepidoptera) and comparison with diapause induction. *J. comp. Physiol. A* **155**, 161-169.
- BUCK, J.B. (1937) Studies on the firefly. I. The effects of light and other agents on flashing of *Photinus pyralis*, with special reference to periodicity and diurnal rhythm. *Physiol. Zool.* **10**, 45-58.
- BUCK, J.B. (1938) Synchronous rhythmic flashing of fireflies. *Quart. Rev. Biol.* **13**, 301-314.
- BUCK, J. (1988) Synchronous rhythmic flashing of fireflies. II. *Quart. Rev. Biol.* **63**, 265-289.
- BUCK, J. and BUCK, E. (1968) Mechanism of rhythmic synchronous flashing of fireflies. *Science* **159**, 1319-1327.
- BUCK, J., BUCK, E., HANSON, F.E., CASE, J.F., METS, L. and ATTA, G.J. (1981a) Control of flashing in fireflies. IV. Free run pacemaking in a synchronic *Pteroptyx*. *J. Comp. Physiol.* **144**, 277-286.
- BUCK, J., BUCK, E., CASE, J.F. and HANSON, F.E. (1981b) Control of flashing in fireflies. V. Pacemaker synchronization in *Pteroptyx cribellata*. *J. Comp. Physiol.* **144**, 287-298.
- BULL, D. L. and ADKISSON, P. L. (1960) Certain factors influencing diapause in the pink bollworm, *Pectinophora gossypiella*. *J. econ. Ent.* **53**, 793-798.
- BULL, D. L. and ADKISSON, P. L. (1962) Fat content of the larval diet as a factor influencing diapause and growth rate of the pink bollworm. *Ann. ent. Soc. Am.* **55**, 499-502.
- BÜNNING, E. (1935) Zur Kenntniss der endogenen Tagesrhythmik bei Insekten und Pflanzen. *Ber. dt. bot. Ges.* **53**, 594-623.
- BÜNNING, E. (1936) Die endogene Tagesrhythmik als Grundlage der Photoperiodischen Reaktion. *Ber. dt. bot. Ges.* **54**, 590-607.
- BÜNNING, E. (1959) Zur Analyse des Zeitsinnes bei *Periplaneta americana*. *Z. Naturf.* **14b**, 1-4.
- BÜNNING, E. (1960) Circadian rhythms and time measurement in photoperiodism. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 249-256.
- BÜNNING, E. (1964) *The Physiological Clock*, 1st English edition. Springer-Verlag.
- BÜNNING, E. 1967 *The Physiological Clock* Revised second edition. Longmans Springer-Verlag New York 167 pp.
- BÜNNING, E. (1969) Common features of photoperiodism in plants and animals. *Photochem. Photobiol.* **9**, 219-228.
- BÜNNING, E. (1973) *The Physiological Clock*, Revised Third Edition. The English Universities Press Ltd., London. 258 pp.
- BÜNNING, E. and JOERRENS, G. (1960) Tagesperiodische antagonistische Schwankungen der Blau-violett und Gelbrot-Empfindlichkeit als Grundlage der photoperiodischen Diapause-Induktion bei *Pieris brassicae*. *Z. Naturf.* **15**, 205-213.
- BÜNNING, E. and MÜLLER, D. (1962) How do organisms measure lunar cycles? *Z. Naturforsch.* **16b**, 391-395.
- BÜNSOW, R. C. (1953) Über Tages- und Jahresrhythmische Änderungen der Photoperiodischen Lichterempfindlichkeit bei *Kalanchoe blossfeldiana* und ihre Beziehungen zur endogenen Tagesrhythmik. *Z. Bot.* **41**, 257-276.
- BUTLER, B., CHANDRASHEKARAN, M. K. and ENGELMANN, W. (1977) Longevity and rate of eclosion of *Drosophila pseudoobscura* flies following a disturbance of the circadian rhythm. *J. interdiscipl. Cycle Res.* **8**, 384.
- BUTTEL-REEPEN, H. von (1900) Sind die Bienen 'Reflexmaschinen'? *Biol. Zbl.* **20**, 97.
- CALDAROLA, P. C. and PITTENDRIGH, C. S. (1974) A test of the hypothesis that D₂O affects circadian oscillations by diminishing the apparent temperature. *Proc. Natn. Acad. Sci., U.S.A.* **71**, 4386-4388.
- CALDWELL, R. L. (1974) A comparison of the migratory strategies of two milkweed bugs, *Oncopeltus fasciatus* and *Lygaeus kalmii*. In: *Experimental Analysis of Insect Behavior* (Ed. BARTON-BROWNE, L.). Springer-Verlag.
- CALLAHAN, P. S. (1958) Behavior of the imago of the corn earworm, *Heliothis zea* (Boddie), with special reference to emergence and reproduction. *Ann. ent. Soc. Am.* **51**, 271-283.
- CALVEZ, B. (1976) Taux d'ecdysone circulante aux derniers ages larvaires et induction de la diapause nymphales chez *Pieris brassicae*. *C.R. Acad. Sci., Paris*, series D, **282**, 1367-1370.
- CAMPBELL, G. D. (1976) Activity rhythm in the cave cricket, *Ceuthophilus conicaudus* Hubbell. *Am. Midl. Nat.* **96**, 350-366.
- CASE, J.F. (1984) Vision in mating behaviour of fireflies. *Symp. Roy. Ent. Soc. London* **12**, 195-222.

- CASE, J.F. and BUCK, J. (1963) Control of flashing in fireflies. II. Role of central nervous system. *Biol. Bull.* **125**, 234-250.
- CASE, J.F. and TRINKLE, M.S. (1968) Light-inhibition of flashing in the firefly, *Photuris missouriensis*. *Biol. Bull.* **135**, 476-485.
- CASTROVILLO, P.J. and CARDE, R.T. (1979) Environmental regulation of female calling and male pheromone response periodicities in the codling moth (*Laspeyresia pomonella*). *J. Insect Physiol.* **25**, 659-668.
- CAUSSE, R. (1974) Etude d'un rythme circadien du comportement du prénymphe chez *Ceratitis capitata* Wiedemann (Diptère Trypetidae). *Ann. Zool. Ecol. animale* **6**, 475-498.
- CHANDRASHEKARAN, M. K. (1967a) Studies on phase-shifts in endogenous rhythms. I. Effects of light pulses on the eclosion rhythm in *Drosophila pseudoobscura*. *Z. vergl. Physiol.* **56**, 154-162.
- CHANDRASHEKARAN, M. K. (1967b) Studies on phase-shifts in endogenous rhythms. II. The dual effect of light on the entrainment of the eclosion rhythm in *Drosophila pseudoobscura*. *Z. vergl. Physiol.* **56**, 163-170.
- CHANDRASHEKARAN, M. K. (1974) Phase shifts in the *Drosophila pseudoobscura* circadian rhythm evoked by temperature pulses of varying length. *J. interdiscipl. Cycle Res.* **5**, 371-380.
- CHANDRASHEKARAN, M.K. (1980) Apparent absence of a separate B-oscillator in phasing the circadian rhythm of eclosion in *Drosophila pseudoobscura*. In: "Development and Neurobiology of *Drosophila*", eds: SIDDIQUI, O., BABU, P., HALL, L.M. and HALL, J.C. Plenum Publishing Co.
- CHANDRASHEKARAN, M. K. and ENGELMANN, W. (1976) Amplitude attenuation of the circadian rhythm in *Drosophila* with light pulses of varying irradiance and duration. *Int. J. Chronobiol.* **3**, 231-240.
- CHANDRASHEKARAN, M. K. and ENGELMANN, W. (1973) Early and late subjective night phases of the *Drosophila pseudoobscura* circadian rhythm require different energies of blue light for phase shifting. *Z. Naturforsch.* **28C**, 750.
- CHANDRASHEKARAN, M. K. and LOHER, W. (1969a) The effect of light intensity on the circadian rhythm of eclosion in *Drosophila pseudoobscura*. *Z. vergl. Physiol.* **62**, 337-347.
- CHANDRASHEKARAN, M. K. and LOHER, W. (1969b) The relationship between the intensity of the light pulses and the extent of phase shifts of the circadian rhythm in the eclosion rate of *Drosophila pseudoobscura*. *J. exp. Zool.* **172**, 147-152.
- CHANDRASHEKARAN, M. K., JOHNSON, A. and ENGELMANN, W. (1973) Possible 'dawn' and 'dusk' roles of light pulses shifting the phase of a circadian rhythm. *J. Comp. Physiol.* **82**, 347-356.
- CHAPMAN, R.F. (1998) *The Insects. Structure and Function*. University Press, Cambridge.
- CHARLTON, R.E. and CARDE, R.T. (1982) Rate and diel periodicity of pheromone emission from female gypsy moths, (*Lymantria dispar*) determined with a glass-adsorption collection system. *J. Insect Physiol.* **28**, 423-430.
- CHEN, D.-M., CHRISTIANSON, J.S., SAPP, R.J. and STARK, W.S. (1992) Visual receptor cycle in normal and period mutant *Drosophila*: microspectrophotometry, electrophysiology, and ultrastructural morphometry. *Vis. Neurosci.* **9**, 125-135.
- CHERBAS, P. and CHERBAS, L. (1996) Molecular aspects of ecdysteroid hormone action. In: Gilbert, L.I., TATA, J.R. and ATKINSON, B.G. (Eds.). *Metamorphosis: Post-embryonic Reprogramming of Gene Expression in amphibian and Insect Cells*. Academic Press, San Diego, pp. 175-221.
- CHEUNG, I.L., FACCIPONTE, G. and LANGE, A.B. (1994) The effects of octopamine on neuromuscular transmission in the oviducts of *Locusta*. *Biogenic amines* **10**, 519-534.
- CHIBA, Y. (1966) The diurnal activity of the mosquito, *Culex pipiens pallens*, in relation to light conditions. IV. The developmental stage at which the physiological 24-hr rhythm is initiated. *Sci. Rep. Tôhoku Univ. IV (Biol)*, **32**, 197-200.
- CHIBA, Y. and TOMIOKA, K. (1987) Insect circadian activity with special reference to the localization of the pacemaker. *Zool. Sci.* **4**, 945-954.
- CHIBA, Y., UKI, M., KAWASAKI, Y., MATSUMOTO, A. and TOMIOKA, K. (1993) Entrainability of circadian activity of the mosquito *Culex pipiens pallens* to 24-hr temperature cycles, with special reference to involvement of multiple oscillators. *J. Biol. Rhythms* **8**, 211-220.
- CHIPPENDALE, G. M. (1977) Hormonal regulation of larval diapause. *Ann. Rev. Ent.* **22**, 121-138.
- CHIPPENDALE, G.M. (1984) Environmental signals, the neuroendocrine system, and the regulation of larval diapause in the southwestern corn borer, *Diatraea grandiosella*. In: *Photoperiodic Regulation of Insect and Molluscan Hormones, Ciba Foundation Symposium* **104**, 259-276.
- CHIPPENDALE, G. M. and REDDY, A. S. (1973) Temperature and photoperiodic regulation of diapause of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect Physiol.* **19**, 1397-1408.
- CHIPPENDALE, G. M., REDDY, A. S. and CATT, C. L. (1976) Photoperiodic and thermoperiodic interactions in the regulation of the larval diapause of *Diatraea grandiosella*. *J. Insect Physiol.* **22**, 823-828.

- CHIPPENDALE, G. M. and YIN, C-M. (1973) Endocrine activity retained in diapause insect larvae. *Nature, Lond.* **246**, 511-513.
- CHOI, M.Y., TATSUKI, S. and BOO, K.S. (1998a) Regulation of sex pheromone biosynthesis in the oriental budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* **44**, 653-658.
- CHOI, M.Y., TANAKA, M., KATAOKA, H., BOO, K.S. and TATSUKI, S. (1998b) Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol.* **28**, 759-766.
- CHRISTENSEN, N.D. (1978) The circadian clock of *Hemideina thoracica* as a population of coupled oscillators. M.Sc. thesis, University of Auckland, New Zealand. 239 pp.
- CHRISTENSEN, N.D. and LEWIS, R.D. (1982) The circadian locomotor rhythm of *Hemideina thoracica* (Orthoptera; Stenopelmatidae): the circadian clock as a population of interacting oscillators. *Physiol. Entomol.* **7**, 1-13.
- CHRISTENSEN, N.D. and LEWIS, R.D. (1983) The circadian locomotor rhythm of *Hemideina thoracica* (Orthoptera; Stenopelmatidae): A population of weakly coupled feedback oscillators as a model of the underlying pacemaker. *Biol. Cybern.* **47**, 165-172.
- CHRISTENSEN, N. D., LEWIS, R.D. and GANDER, P.H. (1984) Properties of a feedback model for the circadian clock of *Hemideina thoracica* (Orthoptera; Stenopelmatidae). *Biol. Cybern.* **51**, 87-92.
- CHRISTENSEN, T.A. and CARLSON, A.D. (1981) Symmetrically organized dorsal unpaired median (DUM) neurons and flash control in the male firefly, *Photuris versicolor*. *Develop. Biology* **93**, 133-147
- CHRISTENSEN, T.A., ITAGAKI, H., TEAL, P.E.A., JASENSKY, R.D., TUMLINSON, J.H. and HILDEBRAND, J.G. (1991) Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths. *Proc. Nat. Acad. Sci. USA* **88**, 4971-4975.
- CHRISTENSEN, T.A., LASHBROOK, J.M. and HILDEBRAND, J.G. (1994) Neural activation of the sex-pheromone gland in the moth *Manduca sexta*: real-time measurement of pheromone release. *Physiol. Entomol.* **19**, 265-270.
- CHRISTENSEN, T.A., LEHMAN, H.K., TEAL, P.E.A., ITAGAKI, H., TUMLINSON, J.H. and HILDEBRAND, J.G. (1992) Diel changes in the presence and physiological actions of octopamine in the female sex-pheromone glands of *Heliothis* moths. *Insect Biochem. Mol. Physiol.* **22**, 841-849.
- CHURCH, N. S. (1955) Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. *Can. J. Zool.* **33**, 339-369.
- CLAES, H. and LANG, A. (1947) Die Blütenbildung von *Hyoscyamus niger* in 48 stündigen Licht-Dunkel-Zyklen und in Zyklen mit Aufgeteilten Lichtphasen. *Z. Naturf.* **2**, 56-63.
- CLARET, J. (1966a) Recherche du centre photorécepteur lors de l'induction de la diapause chez *Pieris brassicae* L. *C. R. Acad. Sci.* **262**, 1464-1465.
- CLARET, J. (1966b) Mise en évidence du rôle photorecepteur du cerveau dans l'induction de la diapause chez *Pieris brassicae* (Lepido). *Annls Endocr.* **27**, 311-320.
- CLARET, J. (1972) Sensibilité spectrale des chenilles de *Pieris brassicae* (L.) lors de l'induction photopériodique de la diapause. *C. R. Acad. Sci.* **274**, 1727-1730.
- CLARET, J. (1973) Le domaine de photosensibilité du parasite *Pimpla instigator* F. (Hymenoptère, Ichneumonidae) lors de l'entrée et de la levée photopériodiques de la diapause. *C. R. Acad. Sci.* **276**, 3163-3166.
- CLARET, J. (1985) Two mechanisms in the biological clock of *Pieris brassicae* L.: An oscillator for diapause induction; an hourglass for diapause termination. *Experientia* **41**, 1613-1615.
- CLARET, J. (1989) Vitamine A et induction photoperiodique ou thermoperiodique de la diapause chez *Pieris brassicae*. *C.R. Acad. Sci. Paris* **308**, 347-352.
- CLARET, J. and ARPAGHAUS, M. (1994) Evidence for "dawn" and "dusk" hourglasses in *Pimpla* photoperiodic clock. *Experientia* **41**, 1613-1615.
- CLARET, J. and CARTON, Y. (1980) Diapause in a tropical species, *Cothonaspis boulardi* (Parasitic Hymenoptera). *Oecologia* **45**, 32-34.
- CLARET, J., DUMORTIER, B. and BRUNNARIUS, J. (1981) Mise en évidence d'une composante circadienne dans l'horloge biologique de *Pieris brassicae* (Lepidoptera), lors de l'induction photopériodique de la diapause. *C. R. Acad. Sci. Paris*, **292**, 427-430.
- CLARET, J. and VOLKOFF, N. (1992) Vitamin A is essential for two processes involved in the photoperiodic reaction in *Pieris brassicae*. *J. Insect Physiol.* **38**, 569-574.
- CLARIDGE-CHANG, A., WIJNEN, H., NAEF, F., BOOTHROYD, C., RAJEWSKY, N. and YOUNG, M.W. (2001) Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* **32**, 657-671.

- CLARK, S. H. and PLATT, A. P. (1969) Influence of photoperiod on development and larval diapause in the viceroys butterfly *Limnitis archippus*. *J. Insect Physiol.* **15**, 1951-1957.
- CLAY, M. E. and VENARD, C. E. (1972) Larval diapause in the mosquito, *Aedes triseriatus*: effects of diet and temperature on photoperiodic induction. *J. Insect Physiol.* **18**, 1441-1446.
- CLAYTON, D. L. and PAIETTA, J. V. (1972) Selection for circadian eclosion time in *Drosophila melanogaster*. *Science, Wash.* **178**, 994-995.
- CLOPTON, J.R. (1984a) Mosquito circadian and circa-bi-dian flight rhythms: a two oscillator model. *J. comp. Physiol. A* **155**, 1-12.
- CLOPTON, J.R. (1984b) Mosquito circadian flight rhythms: differential effects of constant light. *Am. J. Physiol.* **247**, R 960-967.
- CLOPTON, J.R. (1985) Circa-bi-dian rhythmicity in the flight activity of the mosquito *Culiseta incidens*. *Comp. Biochem. Physiol.* **80A**, 469-475.
- CLOUDSLEY-THOMPSON, J. L. (1953) Studies on diurnal rhythms. III. Photoperiodism in the cockroach *Periplaneta americana* (L.). *Ann. Mag. Nat. Hist.* **6**, 705-712.
- CLOUTIER, E. J., BECK, S. D., McLEOD, D. G. R. and SILHACEK, D. L. (1962) Neural transplants and insect diapause. *Nature, Lond.* **195**, 1222-1224.
- CLYNE, P., GRANT, A., O'CONNELL, R. and CARLSON, J.R. (1997) Odorant response of individual sensilla on the *Drosophila* antenna. *Invert. Neurosci.* **3**, 127-135.
- COLWELL, C.S. and PAGE, T.L. (1990) A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. *J. comp. Physiol. A* **166**, 643-649.
- CONSTANTINOU, C. (1984) Circadian rhythm of oviposition in the blood sucking bugs, *Triatoma phyllosoma*, *T. infestans* and *Panstrongylus megistus* (Hemiptera: Reduviidae). *J. interdiscipl. Cycle Res.* **15**, 203-212.
- COOK, R.M. (1973) Courtship processing in *Drosophila melanogaster*. *Anim. Behav.* **21**, 349-358.
- CORBET, P. S. (1955) A critical response to changing length of day in an insect. *Nature, Lond.* **175**, 338-339.
- CORBET, P. S. (1956) Environmental factors influencing the induction and termination of diapause in the emperor dragonfly, *Anax imperator* Leach (Odonata: Aeschnidae). *J. exp. Biol.* **33**, 1-14.
- CORBET, P. S. (1958) Lunar periodicity of aquatic insects in Lake Victoria. *Nature, Lond.* **182**, 330-331.
- CORNELIUS, G. and RENSING, L. (1982) Can phase response curves of various treatments of circadian rhythms be explained by effects on protein synthesis and degradation? *BioSystems* **15**, 35-47.
- COX, P.D. (1979) The influence of photoperiod on the life cycle of *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae). *J. Stored Prod. Res.* **15**, 111-115.
- CUMMING, B. G. (1971) The role of circadian rhythmicity in photoperiodic induction in plants. *Proc. Int. Symp. Circadian Rhythmicity* (Wageningen, 1971), pp. 33-85.
- CUSSON, M. and McNEIL, J.N. (1989) Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. *Science* **243**, 210-212.
- CYMBOROWSKI, B. (1973) Control of the circadian rhythm of locomotor activity in the house cricket. *J. Insect Physiol.* **19**, 1423-1440.
- CYMBOROWSKI, B. (1981) Transplantation of circadian pacemaker in the house cricket, *Acheta domesticus* L. *J. Interdisc. Cycle Res.* **12**, 133-140.
- CYMBOROWSKI, B. (1998) Serotonin modulates a photic response in circadian locomotor rhythmicity of adults of the blow fly, *Calliphora vicina*. *Physiol. Entomol.* **23**, 25-32.
- CYMBOROWSKI, B. and BRADY, J. (1972) Insect circadian rhythms transmitted by parabiosis a re-examination. *Nature, New Biology*, **236**, 221-222.
- CYMBOROWSKI, B. and DUTKOWSKI, A. (1969) Circadian changes in RNA synthesis in the neurosecretory cells of the brain and sub-oesophageal ganglion of the house cricket. *J. Insect Physiol.* **15**, 1187-1197.
- CYMBOROWSKI, B. and DUTKOWSKI, A. (1970) Circadian changes in protein synthesis in the neurosecretory cells of the central nervous system of *Acheta domesticus*. *J. Insect Physiol.* **16**, 341-348.
- CYMBOROWSKI, B., GILLANDERS, S.W., HONG, S.-F. and SAUNDERS, D.S. (1993) Phase shifts of the adult locomotor activity rhythm in *Calliphora vicina* induced by non-steroidal ecdysteroid agonist RH 5849. *J. comp. Physiol.* **172**, 101-108.
- CYMBOROWSKI, B., HONG, S.-F., McWATTERS, H.G. and SAUNDERS, D.S. (1996) S-antigen antibody partially blocks entrainment and the effects of constant light on the circadian rhythm of locomotor activity in the adult blow fly, *Calliphora vicina*. *J. Biol. Rhythms* **11**, 68-74.
- CYMBOROWSKI, B. and KING, V. (1996) Circadian regulation of Fos-like expression in brain of the blow fly, *Calliphora vicina*. *Comp. Biochem. Physiol.* **115C**, 239-246.
- CYMBOROWSKI, B. and KORF, H.-W. (1995) Immunocytochemical demonstration of S-antigen (arrestin) in the brain of the blowfly *Calliphora vicina*. *Cell Tissue Res.* **279**, 109-114.

- CYMBOROWSKI, B., LEWIS, R.D., HONG, S.-F. and SAUNDERS, D.S. (1994) Circadian locomotor activity rhythms and their entrainment to light-dark cycles continue in flies (*Calliphora vicina*) surgically deprived of their optic lobes. *J. Insect Physiol.* **40**, 501-510.
- CYMBOROWSKI, B., MUSZYNSKA-PYTEL, M., PORCHERON, P. and CASSIER, P. (1991) Haemolymph ecdysteroid titres controlled by a circadian clock mechanism in larvae of the wax moth, *Galleria mellonella*. *J. Insect Physiol.* **37**, 35-40.
- CYMBOROWSKI, B., SKANGIEL-KRAMSKA, J. and DUTKOWSKI, A. (1970) Circadian changes of acetylcholinesterase activity in the brain of house-crickets (*Acheta domesticus* L.). *Comp. Biochem. Physiol.* **32**, 367-370.
- CYMBOROWSKI, B., SMIETANKO, A. and DELBECQUE, J.P. (1989) Circadian modulation of ecdysteroid titre in *Galleria mellonella* larvae. *Comp. Biochem. Physiol. A* **94**, 431-438.
- DAAN, S. and BERDE, C. (1978) Two coupled oscillators: simulations of the circadian pacemaker in mammalian activity rhythms. *J. theoret. Biol.* **70**, 297-313.
- DAAN, S. and PITTENDRIGH, C. S. (1976a) A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. *J. comp. Physiol.* **106**, 253-266.
- DAAN, S. and PITTENDRIGH, C. S. (1976b) A functional analysis of circadian pacemaker in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency? *J. comp. Physiol.* **106**, 267-290.
- DAI, J.-D., COSTELLO, M.J. and GILBERT, L.I. (1994) The prothoracic glands of *Manduca sexta*: a microscopic analysis of gap junctions and intercellular bridges. *Invert. Reproduct. Develop.* **25**, 93-110.
- DAI, J.-D., MIZOGUCHI, A., SATAKE, S., ISHIZAKI, H. and GILBERT, L.I. (1995) Developmental changes in the prothoracicotropic hormone content of the *Bombyx mori* brain-retrocerebral complex and haemolymph: Analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Develop. Biol.* **171**, 212-223.
- DANILEVSKII, A. S. (1948) The photoperiodic reaction of insects in conditions of artificial light. *Dokl. Akad. Nauk SSSR*, **60**, 481-484. (In Russian.)
- DANILEVSKII, A. S. (1965) *Photoperiodism and Seasonal Development of Insects*, 1st English edition. Oliver & Boyd, Edinburgh and London.
- DANILEVSKII, A.S. and GLINYANYAYA, E. I. (1949) The effect of the relation between the dark and light periods of the day on insect development. *Dokl. Akad. Nauk SSSR*, **68**, 785-788. (In Russian.)
- DANILEVSKII, A. S., GORYSHIN, N. I. and TYSHCHENKO, V. P. (1970) Biological rhythms in terrestrial arthropods. *A. Rev. Ent.* **15**, 201-244.
- DANKS, H.V. (1987) *Insect Dormancy: An Ecological Perspective*. Biological Survey of Canada (Terrestrial Arthropods), Monograph Series 1, Ottawa.
- DANTHANARAYANA, W. (1986) Lunar periodicity of insect flight and migration. In: *Insect Flight: Dispersal and Migration*, ed: Dathanarayana, W. Springer-Verlag, Heidelberg.
- DARJO, A. (1976) Activité des corpora allata et contrôle photopériodique de la maturation ovarienne chez *Locusta migratoria*. *J. Insect Physiol.* **22**, 347-355.
- DAVEY, K.G. (1985) The male system. In: Kerkut, G.A. and Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Pergamon Press, Oxford, vol. 1, pp. 15-36.
- DAVID, J. and FOUILLET, P. (1973) Enregistrement continue de la ponte chez *Drosophila melanogaster* et importance des conditions expérimentales pour l'étude du rythme circadien d'oviposition. *Rev. comp. Anim.* **7**, 197-202.
- DAVIDSON, J. (1929) On the occurrence of the parthenogenetic and sexual forms of *Aphis rumicis* L. with special reference to the influence of environmental factors. *Ann. appl. Biol.* **16**, 104-134.
- DEDOS, S.G. and FUGO, H. (1999) Induction of dauer larvae by application of fenoxycarb early in the 5th instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **45**, 769-775.
- DE LOOF, A., BAGGERMAN, G., BREUER, M., CLAEYS, I., CERSTIAENS, A., CLYNEN, E., JANSSEN, T., SCHOofs, L. and BROECK, J.V. (2001) Gonadotropins in insects: An overview. *Arch. Insect Biochem. Physiol.* **47**, 129-138.
- DELISLE, J. and McNEIL, J.N. (1987) Calling behaviour and pheromone titre of the true armyworm *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae) under different temperature and photoperiodic conditions. *J. Insect Physiol.* **33**, 315-324.
- DEHN, M. VON (1967) Über den photoperiodismus heterogoner aphiden. Zur frage der Direkten oder indirekten wirkung der tageslange. *J. Insect Physiol.* **13**, 595-612.
- DENLINGER, D.L. (1971) Embryonic determination of pupal diapause in the flesh fly *Sarcophaga crassipalpis*. *J. Insect Physiol.* **17**, 1815-1822.

- DENLINGER, D. L. (1972) Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae). *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **142**, 11-24.
- DENLINGER, D. L. (1974) Diapause potential in tropical flesh flies. *Nature, Lond.* **252**, 223-224.
- DENLINGER, D. L. (1976) Preventing insect diapause with hormones and cholera toxin. *Life Sciences* **19**, 1485-1490.
- DENLINGER, D. L. (1978) The developmental response of flesh flies (Diptera: Sarcophagidae) to tropical seasons. *Oecologia, Berl.* **35**, 105-107.
- DENLINGER, D. L. (1979) Pupal diapause in tropical flesh flies: environmental and endocrine regulation, metabolic rate and genetic selection. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **142**, 31-46.
- DENLINGER, D.L. (1983) Who controls the rhythm of tsetse parturition: mother or larva? *Physiol. Entomol.* **8**, 25-28.
- DENLINGER, D.L. (1985) Hormonal control of diapause. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Eds G.A. Kerkut and L.I. Gilbert, vol. 8, pp 353-412. Pergamon Press, Oxford.
- DENLINGER, D.L. (1986) Dormancy in tropical insects. *Ann. Rev. Entomol.* **31**, 239-264.
- DENLINGER, D.L. (1991) Relationship between cold hardiness and diapause. In: *Insects at Low Temperature*, Eds: Lee, R.E. Jr and Denlinger, D.L., Chapman and Hall, New York. Pp. 174-198.
- DENLINGER, D.L. and BRADFIELD, J.Y. (1981) Duration of pupal diapause in the tobacco hornworm is determined by number of short days received by the larvae. *J. exp. Biol.* **91**, 331-337.
- DENLINGER, D. L., CAMPBELL, J. J. and BRADFIELD, J. Y. (1980) Stimulatory effect of organic solvents on initiating development in diapause pupae of the flesh fly, *Sarcophaga crassipalpis*, and the tobacco hornworm, *Manduca sexta*. *Physiol. Ent.* **5**, 7-15.
- DENLINGER, D.L., JOPLIN, K.H., FLANNAGAN, R.D., TAMMARIELLO, S.P., ZHANG, M.-L., YOCUM, G.D. and LEE, K.-Y. (1995) Diapause-specific gene expression. In: *Molecular Mechanisms of Insect Metamorphosis and Diapause*. Ed: A. Suzuki, H. Kataoka and S. Matsumoto. Industrial Publishing, Tokyo. Pp. 289-297.
- DENLINGER, D. L. and WINGARD, P. (1978) Cyclic GMP breaks pupal diapause in the flesh fly *Sarcophaga crassipalpis*. *J. Insect Physiol.* **24**, 715-719.
- DEPNER, K. R. (1962) Effects of photoperiod and of ultraviolet radiation on the incidence of diapause in the horn fly, *Haematobia irritans* (L.). *Int. J. Bioclimatol. Biometeorol.* **5**, 68-71.
- DICKSON, R. C. (1949) Factors governing the induction of diapause in the oriental fruit moth *Ann. ent. Soc. Am.* **42**, 511-537.
- DINGLE, H. (1972) Migration strategies of insects. *Science, Wash.* **175**, 1327-1335.
- DINGLE, H. (1974) Diapause in a migrant insect, the milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). *Oecologia, Berl.* **17**, 1-10.
- DINGLE, H. (ed.) (1978) *Evolution of Insect Migration and Diapause*. Springer-Verlag, New York.
- DINGLE, H. (1984) Behavior, genes, and life histories: complex adaptations in uncertain environments. In: *A New Ecology: Novel Approaches to Interactive Systems*. Ed: Price, P.W., Slobodchikoff, C.N. and Gaud, W.S. John Wiley & Sons Inc.
- DINGLE, H., BROWN, C. K. and HEGMANN, J. P. (1977) The nature of genetic variance influencing photoperiodic diapause in a migrant insect, *Oncopeltus fasciatus*. *Am. Nat.* **111**, 1047-1059.
- DINGLE, H., CALDWELL, R.L. and HASKELL, J.B. (1969) Temperature and circadian control of cuticle growth in the bug, *Oncopeltus fasciatus*. *J. Insect Physiol.* **15**, 373-378.
- DIXON, A. F. G. (1971) The 'interval timer' and photoperiod in the determination of parthenogenetic and sexual morphs in the aphid, *Drepanosiphum platanoides*. *J. Insect Physiol.* **17**, 251-260.
- DIXON, A. F. G. (1972) The 'interval timer', photoperiod and temperature in the seasonal development of parthenogenetic and sexual morphs in the lime aphid, *Eucallipterus tiliae* L. *Oecologia, Berl.* **9**, 301-310.
- DIXON, A.F.G. (1975) Seasonal changes in fat content, form, state of gonads and length of adult life in the sycamore aphid, *Drepanosiphum platanoides* (Schr.). *Trans. R. Ent. Soc. London* **127**, 87-99.
- DOE, C.Q. and GOODMAN, C.S. (1985) Early events in insect neurogenesis. I. Development and segmental differences in the pattern of neuronal precursor cells. *Develop. Biol.* **111**, 193-205.
- DOLZER, J., KRANNICH, S., FISCHER, K. and STENGL, M. (2001) Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* **204**, 2781-2794.
- DONINI, A., AGRICOLA, H.-J. and LANGE, A.B. (2001) crustacean cardioactive peptide is a modulator of oviduct contractions in *Locusta migratoria*. *J. Insect Physiol.* **47**, 277-285.
- DORN, A., GILBERT, L.I. and BOLLENBACHER, W.E. (1987) Prothoracicotropic hormone activity in the embryonic brain of the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol. B* **157**, 279-283.
- DORTLAND, J. F. (1978) Synthesis of vitellogenins and diapause proteins by the fat body of *Leptinotarsa* as a function of photoperiod. *Physiol. Ent.* **3**, 281-288.

- DOW, J.A.T. and DAVIES, S.A. (2001) The *Drosophila melanogaster* Malpighian tubule. *Adv. Insect Physiol.* **28**, 1-83.
- DOWSE, H.B. (1982) The effects of phase shifts in a 10 Hz electric field cycle on locomotor activity rhythm of *Drosophila melanogaster*. *J. interdiscipl. Cycle Res.* **13**, 257-264.
- DOWSE, H.B., HALL, J.C. and RINGO, J.M. (1987) Circadian and ultradian rhythms in *period* mutants of *Drosophila melanogaster*. *Behavior Genetics* **17**, 19-35.
- DOWSE, H.B. and RINGO, J.M. (1987) Further evidence that the circadian clock in *Drosophila* is a population of coupled ultradian oscillators. *J. Biol. Rhythms* **2**, 65-76.
- DOWSE, H.B. and RINGO, J.M. (1989) Rearing *Drosophila* in constant darkness produces phenocopies of *period* circadian clock mutants. *Physiol. Zool.* **62**, 785-803.
- DOWSE, H.B. and RINGO, J.M. (1993) Is the circadian clock a "metas oscillator?" Evidence from studies of circadian rhythms in *Drosophila*. In *Molecular Genetics of Biological Rhythms*, Young, M.W., ed., Marcel Dekker, New York, pp. 195-220.
- DREISIG, J. (1975) Environmental control of the daily onset of luminescent activity in glowworms and fireflies (Coleoptera: Lampyridae). *Oecologia, Berlin* **18**, 85-99.
- DREISIG, H. (1976) Phase shifting the circadian rhythm of nocturnal insects by temperature changes. *Physiol. Entomol.* **1**, 123-129.
- DREISIG, H. (1978) The circadian rhythm of bio-luminescence in the glow-worm, *Lampyrus noctiluca* L. (Coleoptera: Lampyridae). *Behav. Ecol. Sociobiol.* **3**, 1-18.
- DRESCHER, K., CORNELIUS, G. and RENSING, L. (1982) Phase response curves obtained by observing different variables of a 24 h model oscillator based on translational control. *J. theoret. Biol.* **94**, 345-353.
- DROOP, B. (1975) The effects of altering the length of larval development, at a constant temperature, on photoperiodic induction of diapause in *Sarcophaga argyrostoma*. B.Sc. thesis, University of Edinburgh.
- DUMORTIER, B. (1972) Photoreception in the circadian rhythm of stridulatory activity in *Ephippiger* (Ins., Orthoptera). Likely existence of two photoreceptive systems. *J. comp. Physiol.* **77**, 80-112.
- DUMORTIER, B. (1994) The "circadian paradigm": a test of involvement of the circadian system in the photoperiodic clock. *J. theoret. Biol.* **166**, 101-112.
- DUMORTIER, B. and BRUNNARIUS, J. (1977a) L'information thermopériodique et l'induction de la diapause chez *Pieris brassicae*. *C. R. Acad. Sci. Paris, D*, **284**, 957-960.
- DUMORTIER, B. and BRUNNARIUS, J. (1977b) Existence d'un composante circadienne dans l'induction thermopériodique de la diapause chez *Pieris brassicae* L. *C. R. Acad. Sci. Paris, D*, **285**, 361-364.
- DUMORTIER, B. and BRUNNARIUS, J. (1981) Involvement of the circadian system in photoperiodism and thermoperiodism in *Pieris brassicae* (Lepidoptera). In *Biological Clocks in Seasonal Reproductive Cycles*, Ed. B.K. Follett and D.E. Follett. Bristol, Sciencetechnica, pp. 83-99.
- DUMORTIER, B. and BRUNNARIUS, J. (1987) Photoperiodic time measurement in an insect: is the photoinducible phase ϕ , a reality? In: *Circadian Rhythms, Photoperiod and Endocrine Secretion*. Ed P. Pevet *Comparative Physiology of Environmental Adaptations*, vol 3. Pp 1-22. Karger, Basle.
- DUMORTIER, B. and BRUNNARIUS, J. (1989) Diet-dependent switch from circadian to hourglass-like operation of an insect photoperiodic clock. *J. Biol. Rhythms* **4**, 481-490.
- DUMSER, J.B. (1980) The regulation of spermatogenesis in insects. *Ann. Rev. Entomol.* **25**, 341-369.
- DUNKELBLUM, E., KEHAT, M., HAREL, M. and GORDON, D. (1987) Sexual behaviour and pheromone titre of the *Spodoptera littoralis* female moth. *Ent. Experimentalis et Applicata* **44**, 241-248.
- DUNLAP, J.C. (1999) Molecular bases for circadian clocks. *Cell* **96**, 271-290.
- DUNLAP, J.C., TAYLOR, W. and HASTINGS, J.W. (1980) The effects of protein synthesis inhibitors on the *Gonyaulax* clock. *J. comp. Physiol.* **138**, 1-8.
- DUPORTETS, L., GADENNE, C., DUFOUR, M.C. and COUILLAUD, F. (1998) The pheromone biosynthesis activating neuropeptide (PBAN) of the black curworm moth, *Agrotis ipsilon*: immunohistochemistry, molecular characterization and bioassay of its peptide sequence. *Insect Biochem. Mol. Biol.* **28**, 591-599.
- DUSHAY, M.S., KONOPKA, R.J., ORR, D., GREENACRE, M.L., KYRIACOU, C.P. ROSBASH, M. and HALL, J.C. (1990) Phenotypic and genetic analysis of *Clock*, a new circadian rhythm mutant in *Drosophila melanogaster*. *Genetics* **125**, 557-578.
- DUSHAY, M.S., ROSBASH, M. and HALL, J.C. (1989) The *disconnected* visual system mutations in *Drosophila* drastically disrupt circadian rhythms. *J. Biol. Rhythms* **4**, 1-27.
- DUTKOWSKI, A., CYMBOROWSKI, B. and PRZELECKA, A. (1971) Circadian changes in the ultrastructure of the neurosecretory cells of the pars intercerebralis of the house cricket. *J. Insect Physiol.* **17**, 1763-1772.
- DYSON-HUDSON, V. R. D. (1956) The daily activity rhythm of *Drosophila subobscura* and *D. obscura*. *Ecology*, **37**, 562-567.

- EATON, J.L., TIGNOR, K.R. and HOLTZMAN G.I. (1983) Role of moth ocelli in timing flight initiation at dusk. *Physiol. Entomol.* **8**, 371-375.
- EDMUNDS, L.N. Jr. (1988) *Cellular and Molecular Bases of Biological Clocks: Models and Mechanisms for Circadian Timekeeping*. Springer-Verlag, New York.
- EDRICH, W. (1981) Night-time sun-compass behaviour of honey bees at the equator. *Physiol. Entomol.* **6**, 7-13.
- EDWARDS, R. L. (1954) The host-finding and oviposition behaviour of *Mormoniella vitripennis* (Walker) (Hym., Pteromalidae), a parasite of Muscoid flies. *Behaviour*, **7**, 88-112.
- EERTMOED, G. (1978) Embryonic diapause in the psocid, *Peripsocus quadrfasciatus*: photoperiod, temperature, ontogeny and geographical variation. *Physiol. Ent.* **3**, 197-206.
- EGHTEDAR, E. (1970) Zur Biologie und Ökologie der Staphyliniden *Philonthus fuscipennis* Mannh. und *Oxylelus rugosus* Grav. *Pedobiologia*, **10**, 169-179.
- EIDMANN, H. (1956) Über rhythmische Erscheinungen bei der Stabheuschrecke *Carausius morosus*. *Z. vergl. Physiol.* **28**, 370-390.
- ELEKONICH, M.M., SCHULTZ, D.J., BLOCH, G. and ROBINSON, G.E. (2001) Juvenile hormone levels in honey bee (*Apis mellifera* L.) foragers: foraging experience and diurnal variation. *J. Insect Physiol.* **47**, 119-1125.
- ELLIOT, J.T., JURENKA, R.A., PRESTWICH, G.D. and ROELOFS, W.L. (1997) Identification of soluble proteins for an insect neuropeptide. *Biochem. Biophys. Res. Commun.* **238**, 925-930.
- EMERY, I.F., NOVERAL, J.M., JAMIESON, C.F. and SIWICKI, K.K. (1997) Rhythms of *Drosophila period* gene expression in culture. *Proc. Nat. Acad. Sci. USA* **94**, 4092-4096.
- EMERY, P., STANEWSKY, R., HALL, J.C. and ROSBASH, M. (2000a) *Drosophila* cryptochrome - a unique circadian-rhythm photoreceptor. *Nature* **404**, 456-457.
- ENDO, K. and FUNATSU, S. (1985) Hormonal control of seasonal morph determination in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae). *J. Insect Physiol.* **31**, 669-674.
- ENDO, K., ITO, T. and CHIBA, Y. (1986) Photoperiodic control of pupal diapause in the swallowtail, *Papilio xuthus* L. (Lepidoptera, Papilionidae) – sensitive stage and number of required cycles. *Zool. Sci.* **3**, 351-356.
- ENDO, K. and KANATA, Y. (1985) Hormonal control of seasonal morph determination in the small copper butterfly, *Lycaena phlaeas daimio* Seitz. *J. Insect Physiol.* **31**, 701-706.
- ENDRASS, U. (1976) Physiologische Anpassungen eines marinen Insekts. I. Die zeitliche Steuerung der Entwicklung. *Marine Biology*, **34**, 361-368.
- ENGELMANN, W. (1966) Effect of light and dark pulses on the emergence rhythm of *Drosophila pseudoobscura*. *Experientia*, **22**, 606-608.
- ENGELMANN, W. (1969) Phase shifting of eclosion in *Drosophila pseudoobscura* as a function of energy of light pulse. *Z. vergl. Physiol.* **64**, 111-117.
- ENGELMANN, W. and HONEGGER, H. W. (1966) Tagesperiodischer Schlupfrhythmus einer angelenen *Drosophila melanogaster*-Mutante. *Z. Naturf.* **22B**, 1-2.
- ENGELMANN, W., HELLRUNG, W. and JOHNSSON, A. (1996) Circadian locomotor activity of *Musca* flies: recording method and effect of 10 Hz square wave electric fields. *Bioelectromagnetics* **17**, 100-110.
- ENGELMANN, W. and JOHNSSON, A. (1978) Attenuation of the petal movement rhythm in *Kalanchoe* with light pulses. *Physiol. Plant.* **43**, 68-76.
- ENGELMANN, W. and MACK, J. (1978) Different oscillators control the circadian rhythm of eclosion and activity in *Drosophila*. *J. comp. Physiol.* **127**, 229-237.
- ENGELMANN, W. and SHAPPIRO, D. G. (1965) Photoperiodic control of the maintenance and termination of larval diapause in *Chironomus tentans*. *Nature, Lond.* **207**, 548-549.
- ENRIGHT, J.T. (1980) *The Timing of Sleep and Wakefulness*. Springer, Berlin.
- EPSKY, N.D. and HEATH, R.R. (1993) Pheromone production by male *Anastrepha suspensa* (Diptera: Tephritidae) under natural light cycles in greenhouse studies. *Environ. Entomol.* **22**, 464-469.
- EUSEBIO, E.J. and MOODY, W.J. (1986) Calcium-dependent action potentials in the prothoracic gland of *Manduca sexta*. *J. Exp. Biol.* **126**, 531-636.
- EVANS, W. G. (1976) Circadian and circatidal locomotory rhythms in the intertidal beetle *Thalassidroma barbara* (Horn): Carabidae. *J. exp. Mar. Biol. Ecol.* **22**, 79-90.
- EWER, J., FRISCH, B., HAMBLEN-COYLE, M.J., ROSBASH, M. and HALL, J.C. (1992) Expression of the *period* clock gene within different cells types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* **12**, 3321-3349.
- EWER, J., GAMMIE, S.C. and TRUMAN, J.W. (1997) Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. Exp. Biol.* **200**, 869-881.
- EWER, J. and REYNOLDS, S. (2002) Neuropeptide control of moulting in insects. In: Pfaff, D., Arnold, A.,

- Etgen, A., Fahrbach, S. and Rubin, A.R. (Eds.). *Hormone, Brain and Behaviour*. Academic Press, New York, in press.
- EWER, J., GAMMIE, S.C. and TRUMAN, J.W. (1997) Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. exp. Biol.* **200**, 869-881.
- EWING, A.W. (1988) Cycles in the courtship song of male *Drosophila melanogaster* have not been detected. *Anim. Behav.* **36**, 1091-1097.
- FACCIPONTE, G. and LANGE, A.B. (1992) Characterization of a novel central pattern generator located in the VIIth abdominal ganglion of *Locusta*. *J. Insect Physiol.* **38**, 1011-1022.
- FAN, Y., RAFAELI, A., GILEADI, C. and APPLEBAUM, S.W. (1999) Juvenile hormone induction of pheromone gland PBAN-responsiveness in *Helicoverpa armigera* females. *Insect Biochem. Mol. Biol.* **29**, 635-641.
- FERENZ, H. J. (1975) Photoperiodic and hormonal control of reproduction in male beetles, *Pterostichus nigrata*. *J. Insect Physiol.* **21**, 331-341.
- FERENZ, H. J. (1977) Two-step photoperiodic and hormonal control of reproduction in the female beetle, *Pterostichus nigrata*. *J. Insect Physiol.* **23**, 671-676.
- FIELD, M.D., MAYWOOD, E.S., O'BRIEN, J.A., WEAVER, D.R., REPPERT, S.M. and HASTINGS, M.H. (2000) Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* **25**, 437-447.
- FINGERMAN, M., LAGO, A. D. and LOWE, M. E. (1958) Rhythm of locomotor activity and oxygen consumption of the grasshopper *Romalea microptera*. *Am. Mid. Nat.* **59**, 58-66.
- FINOCCHIARO, L., CALLEBERT, S.G., LAUNAY, J.M. and JALLON, J.M. (1988) Melatonin biosynthesis in *Drosophila*: its nature and its effect. *J. Neurochem.* **50**, 382-387.
- FLEISSNER, G. (1974) Circadian adaptation and pigment migration in the retinula cells of the median eyes of *Androctonus australis* L. (Buthidae, Scorpiones). *J. comp. Physiol.* **91**, 399-416.
- FLEISSNER, G. (1982) Isolation of an insect circadian clock. *J. comp. Physiol.* **149**, 311-316.
- FLEISSNER, G. and FLEISSNER, G. (1992) Feedback loops in the circadian system. In *Circadian Rhythms*, Ed. Zatz, M., Elsevier, Amsterdam. pp. 79-84.
- FLEISSNER, G., FLEISSNER, G. and FRISCH, B. (1993) A new type of putative non-visual photoreceptor in the optic lobe of beetles. *Cell. Tissue Res.* **273**, 435-445.
- FLEUGEL, W. (1978) Oviposition rhythm of individual *Drosophila melanogaster*. *Experientia*, **34**, 65-66.
- FOREL, A. (1910) *Das Sinnesleben der Insekten*. Munich.
- FOSTER, R. and HELFRICH-FÖRSTER, C. (2001) The regulation of circadian clocks by light in fruitflies and mice. *Phil. Trans. R. Soc. Lond. B.*, **356**, 1779-1789.
- FOSTER, S.P. (2000) Periodicity of sex pheromone biosynthesis, release and degradation in the lightbrown apple moth *Epiphyas postvittana* (Walker). *Arch. Insect Biochem. Physiol.* **43**, 125-136.
- FOSTER, W.A. and MORETON, R.B. (1981) Synchronization of activity rhythms with the tide in a saltmarsh collembolan *Anurida maritima*. *Oecologia* **50**, 265-270.
- FRAENKEL, G. and BHASKARAN, G. (1973) Pupariation and pupation in cyclorrhaphous flies (Diptera): terminology and interpretation. *Ann. Ent. Soc. Am.* **66**, 418-422.
- FRAENKEL, G. and HSIAO, C. (1968) Manifestations of a pupal diapause in two species of flies *Sarcophaga argyrostoma* and *S. bullata*. *J. Insect Physiol.* **14**, 689-705.
- FRANK, K. D. and ZIMMERMAN, W. F. (1969) Action spectra for phase shifts of a circadian rhythm in *Drosophila*. *Science, Wash.* **163**, 688-689.
- FRASER, A. (1960) Humoral control of metamorphosis and diapause in the larvae of certain Calliphoridae (Diptera: Cyclorrhapha). *Proc. R. Soc. Edin. B*, **67**, 127-140.
- FRIEDLANDER, M. and REYNOLDS, S.E. (1988) Meiotic metaphases are induced by 20-hydroxyecdysone during spermatogenesis of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* **34**, 1013-1019.
- FRISCH, B. and ASCHOFF, J. (1987) Circadian rhythms in honeybees: entrainment by feeding cycles. *Physiol. Entomol.* **12**, 41-49.
- FRISCH, B., HARDIN, P.E., HAMBLIN-COYLE, M.J., ROSBASH, M. and HALL, J.C. (1994) A promoterless period gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the *Drosophila* nervous system. *Neuron* **12**, 555-570.
- FRISCH, B., FLEISSNER, G., FLEISSNER, G., BRANDES, C. and HALL, J.C. (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock-gene product: labelling of cells with possible importance for the beetle's circadian rhythms. *Cell Tissue Res.* **286**, 411-429.

- FRISCH, B., FLEISSNER, G., FLEISSNER, G. and HALL, J.C. (1996) PER-like immunoreactivity in the brain of the beetle *Pachymorpha sexguttata*: histological characterization of cells with possible importance for the circadian clock system. *Cell Tiss. Res.* **286**, 411-429.
- FRISCH, K. VON (1950) Die Sonne als Kompass im Leben der Bienen. *Experientia*, **6**, 210-221.
- FRISCH, K. VON (1967) *The Dance Language and Orientation of Bees*, English Edition. Belknap Press of Harvard University Press. London: Oxford University Press.
- FRISCH, K. VON and LINDAUER, M. (1954) Himmel und Erde im Konkurrenz bei der Orientierung der Bienen. *Naturwissenschaften*, **41**, 245-253.
- FRYER, G. (1959) Lunar rhythms of emergence, differential behaviour of the sexes, and other phenomena in the African midge, *Chironomus brevivucca* (Kieff.). *Bull. ent. Res.* **50**, 1-8.
- FUGO, H., SAITO, H., NAGASAWA, H. and SUZUKI, A. (1985) Eclosion hormone activity in developing embryos of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **31**, 293-298.
- FUHRER, E. and CHEN, Z. Y. (1979) Effect of photoperiod and temperature on development of the engraver beetle, *Pityogenes chalcographus* L. (Coleoptera, Scolytidae). *Forstwiss. Centralbl.* **98**, 87-90.
- FUJISHITA, M. and ISHIZAKI, H. (1981) Circadian clock and prothoracicotropic hormone secretion in relation to the larval-larval ecdysis rhythm of the saturniid *Samia cynthia ricini*. *J. Insect Physiol.* **27**, 121-128.
- FUJISHITA, M. and ISHIZAKI, H. (1982) Temporal organization of endocrine events in relation to the circadian clock during larval-pupal development in *Samia cynthia ricini*. *J. Insect Physiol.* **28**, 77-84.
- FUJISHITA, M., OHNISHI, E. and ISHIZAKI, H. (1982) The role of ecdysteroids in the determination of gut-purge timing in the saturniid, *Samia cynthia ricini*. *J. Insect Physiol.* **28**, 961-967.
- FUKUDA, S. (1951) Production of the diapause eggs by transplanting the sub-oesophageal ganglion in the silkworm. *Proc. Imp. Acad. Japan*, **27**, 672-677.
- FUKUDA, S. (1963) Déterminisme hormonale de la diapause chez le ver à soie. *Bull. Soc. Zoo. Fr.* **88**, 151-179.
- FUKUDA, S. and TAKEUCHI, S. (1976a) Diapause-factor producing cells in the suboesophageal ganglion of the silkworm, *Bombyx mori* L. *Proc. Jap. Acad.* **43**, 51-56.
- FUKUDA, S. and TAKEUCHI, S. (1976b) Studies on the diapause-factor producing cells in the suboesophageal ganglion of the silkworm, *Bombyx mori* L. *Embryologia* **9**, 333-353.
- FUZEAU-BRAESCH, S. (1966) L'étude de la diapause de *Gryllus campestris* (Orthoptera). *J. Insect Physiol.* **12**, 449-455.
- GADENNE, C. and ANTON, S. (2000) Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. *J. Insect Physiol.* **46**, 1195-1206.
- GADENNE, C., RENOU, M. and SRENG, L. (1993) Hormonal control of pheromone responsiveness in the male black curworm, *Agrotis ipsilon*. *Experientia* **49**, 721-724.
- GAINER, H. (1972) Effects of experimentally induced diapause on the electrophysiology and protein synthesis of identified molluscan neurones. *Brain Res.* **39**, 387-402.
- GAMMIE, S.C. and TRUMAN, J.W. (1997) Neuropeptide hierarchies and the activation of sequential motor behaviours in the hawkmoth, *Manduca sexta*. *J. Neuroscience* **17**, 4389-4397.
- GAMMIE, S.C. and TRUMAN, J.W. (1999) Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. *J. Exp. Biol.* **202**, 343-352.
- GANDER, P. H. (1976) A model for the circadian pacemaker of *Hemideina thoracica* derived from the effects of temperature on its activity rhythm. M.Sc. thesis, University of Auckland, New Zealand.
- GANDER, P.H. (1979) The circadian locomotor activity rhythm of *Hemideina thoracica* (Orthoptera): the effects of temperature perturbations. *J. Int. Chronobiol.* **6**, 243-262.
- GANDER, P.H. and LEWIS, R.D. (1979) The circadian locomotor activity rhythm of *Hemideina thoracica* (Orthoptera): A feedback model for the underlying clock oscillation. *Int. J. Chronobiol.* **6**, 263-280.
- GAO, N. and HARDIE, J. (1997) Melatonin and the pea aphid, *Acyrtosiphon pisum*. *J. Insect Physiol.* **43**, 615-620.
- GAO, N., VON SCHANTZ, M., FOSTER, R.G. and HARDIE, J. (1999) The putative brain photoperiodic photoreceptors in the vetch aphid, *Megoura viciae*. *J. Insect Physiol.* **45**, 1011-1019.
- GARNER, W. W. and ALLARD, H. A. (1920) Effect of the relative length of the day and night and other factors of the environment on growth and reproduction in plants. *J. Agric. Res.* **18**, 553-606.
- GEISLER, M. (1961) Untersuchungen zur Tagesperiodik des Mistkäfers *Geotrupes silvaticus* Panz. *Z.Tierpsychol.* **18**, 389-420.
- GEISPITZ, K. F. (1953) The reaction of univoltine Lepidoptera to day-length. *Ent. Obozr.* **33**, 17-31. (in Russian.)

- GEISPITZ, K. F. (1957) The mechanism of acceptance of light stimuli in the photoperiodic reaction of Lepidoptera larvae. *Zool. Zh.* **36**, 548-560. (In Russian.)
- GEISPITZ, K. F. (1965) *Ent. Obozr.* **44**, 538.
- GEISPITZ, K. F. and KYAO, N. N. (1953) The effect of the duration of light on the development of some ichneumonids (Hymenoptera, Braconidae). *Ent. Obozr.* **33**, 32-35. (In Russian.)
- GELDIAY, S. (1967) Hormonal control of adult reproductive diapause in the Egyptian grasshopper, *Anacridium aegyptium* L. *J. Endocr.* **37**, 63-71
- GELDIAY, S. (1971) Control of adult reproductive diapause in *Anacridium aegyptium* L. by direct action of photoperiod on the cerebral neurosecretory cells. Proc.XIIIth. Congr. Ent. Moscow, 1968, **1**, 379-380.
- GELMAN, D.B., WOODS, C.W. and BORKOVEC, A.B. (1988) Effects of ecdysone and 20-hydroxyecdysone on apyrene spermiogenesis in European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **34**, 733-738.
- GELMAN, D.B., THYAGARAJA, B.S., KELLY, T.J., MASLER, E.P., BELL, R.A. and BORKOVEC, A.B. (1992) Prothoracicotropic hormone levels in brains of the European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **38**, 383-395.
- GEMENO, C. and HAYNES, K.F. (2000) Periodical and age-related variation in chemical communication system of black cutworm moth, *Agrotis ipsilon*. *J. Chemical Ecol.* **26**, 329-342.
- GHIRADELLA, H. (1977) Fine structure of the tracheoles of the lantern of the photurid firefly. *J. Morph.* **153**, 187-204.
- GIBBS, D. (1975) Reversal of pupal diapause in *Sarcophaga argyrostoma* by temperature shifts after puparium formation. *J. Insect Physiol.* **21**, 1179-1186.
- GIEBULTOWICZ, J.M. (1999) Insect circadian clocks: is it all in their heads? *J. Insect Physiol.* **45**, 791-800.
- GIEBULTOWICZ, J.M. (2000) Molecular mechanism and cellular distribution of insect circadian clocks. *Ann. Rev. Entomol.* **45**, 767-791.
- GIEBULTOWICZ, J.M. and HEGE, D.M. (1997) Circadian clock in Malpighian tubules. *Nature* **386**, 664.
- GIEBULTOWICZ, J.M., BLACKBURN, M.B., THOMAS-LAEMONT, P.A., WEYDA, F. and RAINA, A.K. (1996) Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. *J. comp. Physiol. A* **178**, 629-636.
- GIEBULTOWICZ, J.M. and BROOKS, N.L. (1998) The circadian rhythm of sperm release in the codling moth, *Cydia pomonella*. *Ent. Experimentalis et Applicata* **88**, 229-234.
- GIEBULTOWICZ, J.M., FELDLAUFER, M. and GELMAN, D.B. (1990) Role of ecdysteroids in the regulation of sperm release from the testis of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* **36**, 567-571.
- GIEBULTOWICZ, J.M., IVANCHENKO, M. and VOLLINTINE, T. (2001) Organization of the insect circadian system: spatial and developmental of clock genes in peripheral tissues of *Drosophila melanogaster*. In *Insect Timing: Circadian Rhythmicity and Seasonality*, Denlinger, D.L., Giebultowicz, J.M., and Saunders, D.S., eds., Elsevier, Amsterdam, pp 31-42.
- GIEBULTOWICZ, J.M. and JOY, J.E. (1992) Ontogeny of the circadian system controlling release of sperm from the insect testis. *J. Biol. Rhythms* **7**, 203-212.
- GIEBULTOWICZ, J.M., JOY, J.E., RIEMANN, J.G. and RAINA, A.K. (1994) Changes in protein patterns in sperm and vas deferens during the daily rhythm of sperm release in the gypsy moth. *Arch. Insect Biochem. Physiol.* **27**, 65-75.
- GIEBULTOWICZ, J.M., RIDGEWAY, R.L. and IMBERSKI, R.B. (1990) Physiological basis for sterilizing effects of constant light in *Lymantria dispar*. *Physiol. Entomol.* **15**, 149-156.
- GIEBULTOWICZ, J.M., RIEMANN, J.G., RAINA, A.K. and RIDGEWAY, R.L. (1989) Circadian system controlling release of sperm in the insect testis. *Science* **245**, 1098-1100.
- GIEBULTOWICZ, J.M., STANEWSKY, R., HALL, J.C. and HEGE, D.M. (2000) Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr. Biol.* **10**, 107-110.
- GIEBULTOWICZ, J.M., WEBB, R.E., RAINA, A.K. and RIDGEWAY, R.L. (1992) Effects of temperature and age on daily changes in pheromone titre in laboratory-reared and wild gypsy moth (Lepidoptera: Lymantriidae). *Environ. Entomol.* **21**, 822-826.
- GIEBULTOWICZ, J.M., WEYDA, F., ERBE, E.F. and WERGIN, W.P. (1997) Circadian rhythm of sperm release in the gypsy moth, *Lymantria dispar*: ultrastructural study of transepithelial penetration of sperm bundles. *J. Insect Physiol.* **43**, 1133-1147.
- GIEBULTOWICZ, J.M. and ZDAREK, J. (1996) The rhythms of sperm release from testes and mating flight are not correlated in *Lymantria* moths. *J. Insect Physiol.* **42**, 167-170.
- GILBERT, L. I., BOLLENBACHER, W. E. and GRANGER, N. A. (1980) Insect Endocrinology: Regulation of endocrine glands, hormone titer, and hormone metabolism. *Ann. Rev. Physiol.* **42** 493-510.
- GILBERT, L.I., RYBCZYNSKI, R. and TOBE, S.S. (1996) Endocrine cascade in insect metamorphosis.

- In: Gilbert, L.I., Tata, J.R. and Atkinson, B.G. (Eds.). *Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. Academic press, San Diego, pp. 59-107.
- GILLET, J. D. (1962) Contributions to the oviposition cycle by the individual mosquitoes in a population. *J. Insect Physiol.* **8**, 665-681.
- GILLET, J. D. (1972) *The Mosquito. Its Life, activities, and Impact on Human Affairs*. Doubleday & Co. Inc., Garden City, N.Y.
- GILLET, J. D., CORBET, P. S. and HADDOW, A. J. (1961) Observations on the oviposition-cycle of *Aedes (Stegomyia) aegypti* (Linnaeus) VI. *Ann. trop. Med. Parasit.* **55**, 427-431.
- GILLET, J. D., HADDOW, A. J. and CORBET, P. S. (1959) Observations on the oviposition-cycle of *Aedes (Stegomyia) aegypti* (Linnaeus) V. *Ann. trop. Med. Parasit.* **53**, 35-41.
- GILLOT, C. (1995) Insect male mating systems. In: Leather, S.R. and Hardie, J. (Eds.), *Insect Reproduction*, CRC Press, London, pp. 33-55.
- GIRAUDGUILLE, M.M., 1996. Twisted liquid crystalline supramolecular arrangements in morphogenesis *Intern. Rev. Cytology-A Survey of Cell Biology* **166**, 59-101.
- GLINYANAYA, Y. I. (1975) The importance of daylength in regulating the seasonal cycles and diapause in some Psocoptera. *Ent. Obozr.* **54**, 10-13.
- GNAGEY, A.L. and DENLINGER, D.L. (1984) Photoperiodic induction of pupal diapause in the flesh fly, *Sarcophaga crassipalpis*: embryonic sensitivity, *J. comp. Physiol. B* **154**, 91-96.
- GODDEN, D. H. (1973) A re-examination of circadian rhythmicity in *Carausius morosus*. *J. Insect Physiol.* **19**, 1377-1386.
- GOLDBETER, A. (1995) A model for circadian oscillations in the *Drosophila period* protein (PER). *Proc. Roy. Soc. Lond. B* **261**, 319-324.
- GOLDBETER, A. (1996) *Biochemical Oscillations and Cellular Rhythms*. Cambridge University Press, Cambridge.
- GOLDSON, S. L. (1981) Reproductive diapause in the Argentine stem weevil (*Listronotus bonariensis* (Kuschel)) (Coleoptera: Curculionidae) in New Zealand. *Bull. Ent. Res.* **71**, 275-287.
- GOMI, T. and TAKEDA, M. (1992) A quantitative photoperiodic response terminates summer diapause in the tailed zygænid moth, *Elcysma westwoodii*. *J. Insect Physiol.* **38**, 665-670.
- GOODWIN, B.C. (1965) Oscillatory behavior in enzymatic control processes. In *Advances in Enzyme Regulation* Vol. 3, (Ed. WEBER, G.), pp. 425-438. Pergamon, Oxford UK.
- GORBET, D. and STEEL, C.G.H. (2002) A circadian rhythm of melatonin in the haemolymph of the insect *Rhodnius prolixus*. Submitted.
- GORYSHIN, N. I. (1955) The relation between light and temperature factors in the photoperiodic reaction in insects. *Ent. Obozr.* **34**, 9-14. (In Russian.)
- GORYSHIN, N. I. (1964) The influence of diurnal light and temperature rhythms on diapause in Lepidoptera. *Ent. Obozr.* **43**, 43-46. (In Russian.)
- GORYSHIN, N. I. and KOZLOVA, R. N. (1967) Thermoperiodism as a factor in the development of insects. *Zhur. obshch. Bio.* **28**, 278-288. (In Russian.)
- GORYSHIN, N. I. and TYSHCHENKO, G. F. (1973) The accumulation of photoperiod information in the cabbage moth *Barathra brassicae* L. (Lepidoptera, Noctuidae) during diapause induction. *Ent. Obozr.* **52**, 249-255. (In Russian)
- GORYSHIN, N. I. and TYSHCHENKO, V. P. (1968) Physiological mechanism of photoperiodic reaction and the problem of endogenous rhythms. In *Photoperiodic Adaptations in Insects and Acari* (Ed. DANILEVSKII, A. S.), pp. 192-269. Leningrad University Press. (In Russian.)
- GORYSHIN, N. I. and TYSHCHENKO, V. P. (1970) Thermostability of the process of perception of photoperiodic information in the moth *Acronycta rumicis* (Lepidoptera, Noctuidae). *Dokl. Akad. Nauk SSSR*, **193**, 458-461. (In Russian.)
- GORYSHIN, N. I. and TYSHCHENKO, V. P. (1974) The place of the memory link in the mechanism of photoperiodic reaction in insects. *Zh. obshch. Biol.* **35**, 518-530. (In Russian.)
- GOSS R. J. (1969a) Photoperiodic control of antler cycles in deer. I. Phase shift and frequency changes. *J. exp. Zool.* **170**, 311-324.
- GOSS, R. J. (1969b) Photoperiodic control of antler cycles in deer. II. Alteration in amplitude. *J. exp. Zool.* **171**, 233-234.
- GRABENBERGER, W. (1934) Experimentelle Untersuchungen über das Zeitgedächtnis von Bienen und Wespen nach Verfütterung von Euchinin und Jodthryeoglobulin. *Z. vergl. Physiol.* **20**, 338-342.
- GREENFIELD, M.D. and PENER, M.P. (1992) Alternative schedules of male reproductive diapause in the grasshopper *Anacridium aegyptium* (L.): Effects of the corpora allata on sexual behavior (Orthoptera: Acrididae). *J. Insect Behav.* **5**, 245-261.

- GREENSPAN, R.J., TONONI, G., CIRELLI, C. and SHAW, P.J. (2001) Sleep and the fruit fly. *Trends Neurosci.* **24**, 142-145.
- GRIENEISEN, M.L. (1994) Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. *Insect Biochem. Mol. Biol.* **24**, 115-132.
- GRUWEZ, G., HOSTE, C., LINTS, C. V. and LINTS, F. A. (1972) Oviposition rhythm in *Drosophila melanogaster* and its alteration by a change in the photoperiodicity. *Experientia*, **27**, 1414-1416.
- GUNN, D. L. (1940) The daily rhythm of activity of the cockroach, *Blatta orientalis*. *J. exp. Biol.* **17**, 267-277.
- GVAKHARIA, B.O., KILGORE, J.A., BEBAS P., and GIEBULTOWICZ, J.M. (2000) Temporal and spatial expression of the *period* gene in the reproductive system of the codling moth. *J. Biol. Rhythms* **15**, 4-12.
- GWINNER, E. (1966) Entrainment of a circadian rhythm in birds by species-specific song cycles (Aves, Fringillidae; *Carduelis spinus*, *Serinus serinus*). *Experientia* **22**, 765.
- GWINNER, E. (1967) Circannuale Periodik der Mauser und der Zugenruhe bei einem Vogel. *Naturwiss.*, **54**, 447.
- GWINNER, E. (1971) A comparative study of circannual rhythms in warblers. In *Biochronometry* (Ed. MENAKER, M.), pp. 405-427. National Academy of Sciences, Washington.
- GWINNER, E. (1974) Testosterone induces 'splitting' of circadian locomotor activity in birds. *Science, Wash.* **185**, 72-74.
- GWINNER, E. (1978) Effects of pinealectomy on circadian locomotor activity rhythms in European starlings, *Sturnus vulgaris*. *J. comp. Physiol.* **126**, 123-129.
- HADDOW, A. J. and GILLET, J. D. (1957) Observations on the oviposition-cycle of *Aedes (Stegomyia) aegypti* (Linnaeus). *Ann. trop. Med. Parasit.* **51**, 159-169.
- HADDOW, A. J., GILLET, J. D. and CORBET, P. S. (1959) Laboratory observations on pupation and emergence in the mosquito *Aedes (Stegomyia) aegypti* (Linnaeus). *Ann. trop. Med. Parasit.* **53**, 123-131.
- HADDOW, A. J., GILLET, J. D. and CORBET, P. S. (1961) Observations on the oviposition-cycle of *Aedes (Stegomyia) aegypti* (Linnaeus) V. *Ann. trop. Med. Parasit.* **55**, 343-356.
- HAGEDORN, H.H. (1985) The role of ecdysteroids in reproduction. In: Kerkut, G.A. and Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. **8**, Pergamon Press, Oxford, pp. 205-262.
- HAGEDORN, H.H. (1989) Physiological roles of haemolymph ecdysteroids in the adult insect. In: Koolman, J. (Ed.) *Ecdysone*, Georg Thieme Verlag, Stuttgart, pp. 279-289.
- HAGSTRUM, D.W. and SILHACEK, D.L. (1980) Diapause induction in *Ephestia cautella*: an interaction between genotype and crowding. *Entomol. Exp. Appl.* **28**, 29-37.
- HALL, J.C. (1995) Tripping along the trail to the molecular mechanisms of biological clocks. *Trends Neurosci.* **18**, 230-240.
- HALL, J.C. (1996) Are cycling gene products as Zeitgebers no longer the Zeitgeist of chronobiology? *Neuron* **17**, 799-802.
- HALL, J.C. (1998a) Genetics of biological rhythms in *Drosophila*. *Adv. Genet.* **38**, 135-184.
- HALL, J.C. (1998b) Molecular neurogenetics of biological rhythms. *J. Neurogenet.* **12**, 115-181.
- HAMBLIN, M., ZEHRING, W.A., KYRIACOU, C.P., REDDY, P., YU, Q., WHEELER, D.A., ZWIEBEL, L.J., KONOPKA, R.J., ROSBASH, M. and HALL, J.C. (1986) Germ-line transformation involving DNA from the *period* locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to *per⁰* and *per⁻* mutants. *J. Neurogenet.* **3**, 249-291.
- HAMBLIN-COYLE, M., KONOPKA, R.J., ZWIEBEL, L.J., COLLOT, H.V., DOWSE, H.B., ROSBASH, M. and HALL, J.C. (1989) A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. *J. Neurogenet.* **5**, 229-256.
- HAMBLIN-COYLE, M.J., WHEELER, D.A., RUTILA, J.E., ROSBASH, M. and HALL, J.C. (1992) Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae). *J. Insect Behav.* **5**, 417-446.
- HAMM, U., CHANDRASHEKARAN, M. K. and ENGELMANN, W. (1975) Temperature-sensitive events between photoreceptor and circadian clock? *Z. Naturforsch.* **30C**, 240-244.
- HAMNER, K. C. (1960) Photoperiodism and circadian rhythms. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 269-277.
- HAMNER, K. C., FLINN, J. C., SIROHI, G. S., HOSHIZAKI, T. and CARPENTER, B. H. (1962) Studies of the biological clock at the south pole. *Nature, Lond.* **195**, 476-480.
- HAMNER, W. M. (1963) Diurnal rhythms and photoperiodism in testicular recrudescence of the house finch. *Science, Wash.* **142**, 1294-1295.
- HAMNER, W. M. (1964) Circadian control of photoperiodism in the house finch demonstrated by interrupted-night experiments. *Nature, Lond.* **203**, 1400-1401.

- HAMNER, W. M. (1969) Hour-glass dusk and rhythmic dawn timers control diapause in the codling moth. *J. Insect Physiol.* **15**, 1499-1504.
- HAN, G., DU, J. and LI, J. (2000) Mating behavioural ecology of *Ancylis sativa* adult. *Yingyong Shengtai Xuebao* **11**, 99-102.
- HAN, SANG-ZIN (1986) Wirkung von Azadirachtin auf die Laufaktivitätsrhythmik der Schabe *Leucophaea maderae* (Fabricius). Ph.D. thesis University of Tuebingen.
- HANDLER, A. M. and KONOPKA, R. J. (1979) Transplantation of a circadian pacemaker in *Drosophila*. *Nature, Lond.* **279**, 236-238.
- HANSON, F.E. (1978) Comparative studies of firefly pacemakers. *Fed. Proc.* **37**, 2158-2164.
- HANSON, F.E. (1982) Pacemaker control of rhythmic flashing of fireflies. In: Carpenter, D.O. (Ed.), *Cellular Pacemakers: Function in Normal and Disease States*, vol. 2, Wiley, New York, pp. 81-100.
- HANSON, F.E., CASE, J.F., BUCK, E. and BUCK, J. (1971) Synchrony and flash entrainment in a New Guinea firefly. *Science* **174**, 161-164.
- HANSTROM, B. (1939) *Hormones in Invertebrates*, Oxford.
- HARCOURT, D. C. and CASS, L. M. (1966) Photoperiodism and fecundity in *Plutella maculipennis* (Curt). *Nature, Lond.* **210**, 217-218.
- HARDELAND, R. and STANGE, G. (1971) Einflüsse von Geschlecht und Alter auf die lokomotorische Aktivität von *Drosophila*. *J. Insect Physiol.* **17**, 427-434.
- HARDELAND, R. and STANGE, G. (1973) Comparative studies on the circadian rhythms of locomotor activity of 40 *Drosophila* species. *J. interdiscipl. Cycle Res.* **4**, 353-359.
- HARDER, R. and BODE, O. (1943) Über die Wirkung von Zwischenbelichtungen während der Dunkelperiode auf das Blühen, die Verlaubung und die Blattsukkulenz bei der Kurztagpflanze *Kalanchoe blossfeldiana*. *Planta*, **33**, 469-504.
- HARDIE, J. (1981a) Juvenile hormone and photoperiodically controlled polymorphism in *Aphis fabae*: prenatal effect on presumptive oviparae. *J. Insect Physiol.* **27**, 257-265.
- HARDIE, J. (1981b) Juvenile hormone and photoperiodically controlled polymorphism in *Aphis fabae*: postnatal effects on presumptive gynoparae. *J. Insect Physiol.* **27**, 347-355.
- HARDIE, J. (1984) A hormonal basis for the photoperiodic control of polymorphism in aphids. In: *Photoperiodic Regulation of Insect and Molluscan Hormones*, Ciba Foundation Symposium **104**, 240-258.
- HARDIE, J. (1987) The corpus allatum, neurosecretion and photoperiodically controlled polymorphism in an aphid. *J. Insect Physiol.* **33**, 201-205.
- HARDIE, J. (1987) The photoperiodic control of wing development in the black bean aphid, *Aphis fabae*. *J. Insect Physiol.* **33**, 543-549.
- HARDIE, J. (1990) The photoperiodic counter, quantitative day-length effects and scotophase timing in the vetch aphid *Megoura viciae*. *J. Insect Physiol.* **36**, 939-949.
- HARDIE, J. (1995) Hormones and reproduction. In: *Insect Reproduction*, eds. Leather, S.R. and Hardie, J. CRC Press, London, pp. 95-108.
- HARDIE, J., BAKER, F.C., JAMIESON, G.C., LEES, A.D. and SCHOOLEY, D.A. (1985) The identification of an aphid juvenile hormone and its titre in relation to photoperiod. *Physiol. Entomol.* **10**, 297-302.
- HARDIN, P.E. (1994) Analysis of period mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. *Mol. Cell. Biol.* **14**, 7211-7218.
- HARDIN, P.E., HALL, J.C. and ROSBASH, M. (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536-540.
- HARDIN, P.E., HALL, J.C. and ROSBASH, M. (1992a) Behavioral and molecular analyses suggest that circadian output is disrupted by disconnected mutants in *D. melanogaster*. *EMBO J.* **11**, 1-6.
- HARDIN, P.E., HALL, J.C. and ROSBASH, M. (1992b) Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. *Proc. Nat. Acad. Sci. US* **89**, 11711-11715.
- HARKER, J. E. (1954) Diurnal rhythm in *Periplaneta americana*. *L. Nature, Lond.* **173**, 689-690.
- HARKER, J. E. (1955) Control of diurnal rhythms of activity in *Periplaneta americana*. *L. Nature, Lond.* **175**, 733.
- HARKER, J. E. (1956) Factors controlling the diurnal rhythm of activity in *Periplaneta americana*. *L. J. exp. Biol.* **33**, 224-234.
- HARKER, J. E. (1958a) Experimental production of midgut tumours in *Periplaneta americana*. *L. J. exp. Biol.* **35**, 251-259.
- HARKER, J. E. (1958b) Diurnal rhythms in the animal kingdom. *Biol. Rev.* **33**, 1-52.
- HARKER, J. E. (1960a) The effect of perturbations in the environmental cycle on the diurnal rhythm of activity of *Periplaneta americana*. *L. J. exp. Biol.* **37**, 154-163.

- HARKER, J. E. (1960b) Internal factors controlling the suboesophageal ganglion neurosecretory cycle in *Periplaneta americana*. *L. J. exp. Biol.* **37**, 164-170.
- HARKER, J. E. (1960c) Endocrine and nervous factors in insect circadian rhythms. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 279-287.
- HARKER, J. E. (1964) *The Physiology of Diurnal Rhythms*. Cambridge University Press, London and New York.
- HARKER, J. E. (1965a) The effect of a biological clock on the development rate of *Drosophila* pupae. *J. exp. Biol.* **42**, 323-337.
- HARKER, J. E. (1965b) The effect of photoperiod on the development rate of *Drosophila* pupae. *J. exp. Biol.* **43**, 411-423.
- HARRIS, F. A., LLOYD, E. P., LANE, H. C. and BURT, E. C. (1969) Influence of light on diapause in the boll weevil. II. Dependence of diapause response on various bands of visible radiation and a broad band of infrared radiation used to extend the photoperiod. *J. econ. Ent.* **62**, 854-857.
- HARRIS, K. M. (1962) Lepidopterous stemborers of cereals in Nigeria. *Bull. ent. Res.* **53**, 139-171.
- HARTLAND-ROWE, R. (1955) Lunar rhythm in the emergence of an Ephemeropteran. *Nature, Lond.* **176**, 657.
- HARTLAND-ROWE, R. (1958) The biology of a tropical mayfly *Povilla adusta* Navas (Ephemeroptera, Polymitarcidae) with special reference to the lunar rhythm of emergence. *Revue Zool. Bot. afr.* **58**, 185-202.
- HARVEY, G. T. (1957) The occurrence and nature of diapause-free development in the spruce budworm, *Choristoneura fumiferana* (Clem.). *Can. J. Zool.* **35**, 549-572.
- HARWOOD, R. F. and HALFHILL, E. (1964) The effect of photoperiod on fat body and ovarian development of *Culex tarsalis*. *Ann. ent. Soc. Am.* **57**, 596-600.
- HARWOOD, R. F. and TAKATA, N. (1965) Effect of photoperiod and temperature on fatty acid composition of the mosquito *Culex tarsalis*. *J. Insect Physiol.* **11**, 711-716.
- HASEGAWA, K. (1951) Studies in voltinism in the silkworm, *Bombyx mori* L., with special reference to the organs concerning voltinism (a preliminary note). *Proc. Imp. Acad. Japan*, **27**, 667-671.
- HASEGAWA, K. and SHIMIZU, I. (1987) *In vivo* and *in vitro* photoperiodic induction of diapause using isolated brain-suboesophageal ganglion complexes of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **33**, 959-966.
- HASEGAWA, K. and SHIMIZU, I. (1988) Occurrence of retinal and 3-hydroxyretinal in a possible photoreceptor of the silkworm brain involved in photoperiodism. *Experientia* **44**, 74-76.
- HASHIMOTO, H. (1966) Discovery of *Clunio takahashii* from Japan. *Jap. J. Zool.* **14**, 13-29.
- HASTINGS, J. W. (1970) Cellular-biochemical clock hypothesis. In: *The Biological Clock: Two Views* (Ed. PALMER, J. D.), pp. 61-91. Academic Press, New York and London.
- HASTINGS, J. W. and KEYNAN, A. (1965) Molecular aspects of circadian systems. In: *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 167-182. North-Holland, Amsterdam.
- HAU, M. and GWINNER, E. (1992) Circadian entrainment by feeding cycles in house sparrows, *Passer domesticus*. *J. comp. Physiol.*, **A 170**, 403-409.
- HAYES, D. K. (1971) Action spectra for breaking diapause and absorption spectra of insect brain tissue. In: *Biochronometry* (Ed. MENAKER, M.), pp. 392-402. National Academy of Sciences, Washington.
- HAYNES, K.F. and BIRCH, M.C. (1984) The periodicity of pheromone release and male responsiveness in the artichoke moth, *Platyptilia carduidactyla*. *Physiol. Entomol.* **9**, 287-296.
- HEDWIG, B. (1995) Neuronal basis of sound generation and sound processing in acridid grasshoppers. *Verh. Dt. Zool. Ges.* **88**, 5-12.
- HEGE, D.M., STANEWSKY, R., HALL, J.C. and GIEBULTOWICZ, J.M. (1997) Rhythmic expression of a PER-reporter in the malpighian tubules of decapitated *Drosophila*: evidence for a brain independent circadian clock. *J. Biol. Rhythms* **12**, 300-308.
- HEIMBACH, F. (1978a) Emergence times of the intertidal midge *Clunio marinus* (Chironomidae) at places with abnormal tides. In: *Physiology and Behaviour of marine Organisms* (Ed. McLUSKY, D. S. and BERRY, A. I.), pp. 263-270. Pergamon Press, Oxford and New York.
- HEIMBACH, F. (1978b) Sympatric species, *Clunio marinus* Hal. and *Cl. balticus* n. sp. (Dipt., Chironomidae) isolated by differences in diel eclosion time. *Oecologia*, **32**, 195-202.
- HEIMBECK, G., BUGNON, V., GENDRE, N., KELLER, A. and STOCKER, R.F. (2001) A central neural for experience-independent olfactory and courtship behaviour in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci., USA* **98**, 15336-15341.
- HEINRICH, R. and ELSNER, N. (1997) Central nervous control of hindleg coordination in stridulating grasshoppers. *J. Comp. Physiol.* **A 180**, 257-269.
- HEINZELLER, T. (1976) Circadiane nderungen im endokrinen System der Honigbiene, *Apis mellifera*, effekt von haft und ocellenblindung. *J. Insect Physiol.* **22**, 315-321.
- HELFRICH, C. (1986) Role of the optic lobes in the regulation of the locomotor activity rhythm of *Drosophila melanogaster*: behavioral analysis of neural mutants. *J. Neurogenetics* **3**, 321-343.

- HELFRICH, C. (1987) Use of *Drosophila melanogaster* brain mutants for the localization of the pacemaker of circadian locomotor activity rhythms. *J. Neurogenetics* **4**, 137-140.
- HELFRICH, C., CYMBOROWSKI, B. and ENGELMANN, W. (1985) Circadian activity rhythm of the house fly continues after optic tract severance and lobectomy. *Chronobiol. Intern.* **2**, 19-32.
- HELFRICH, C. and ENGELMANN, W. (1983) Circadian rhythm of locomotor activity in *Drosophila melanogaster* and its mutants 'sine oculis' and 'small optic lobes'. *Physiol. Entomol.* **8**, 257-272.
- HELFRICH, C. and ENGELMANN, W. (1987) Evidences for circadian rhythmicity in the *per*⁰ mutant of *Drosophila melanogaster*. *Z. Naturforsch.* **42C**, 1335-1338.
- HELFRICH-FÖRSTER, C. (1988) The behaviour of mutant and wildtype flies of *Drosophila melanogaster* under continuous light conditions. *Proc. VI Ann. ESC Frankfurt*.
- HELFRICH-FÖRSTER, C. (1995) The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **92**, 612-616.
- HELFRICH-FÖRSTER, C. (1996) *Drosophila* rhythms: from brain to behavior. *Cell & dev. Biol.* **7**, 791-802.
- HELFRICH-FÖRSTER, C. (1997a) Photic entrainment of *Drosophila*'s activity rhythm occurs via retinal and extraretinal pathways. *Biol. Rhythms Res.* **28**(Suppl.), 119.
- HELFRICH-FÖRSTER, C. (1997b) Development of pigment dispersing hormone immunoreactive neurons in the nervous system of *Drosophila melanogaster*. *J. comp. Neurol.* **380**, 335-354.
- HELFRICH-FÖRSTER, C. (1998) Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of *disconnected* mutants. *J. comp. Physiol. A* **182**, 435-453.
- HELFRICH-FÖRSTER, C. (2000) Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster* – sex specific differences suggest a different quality of activity. *J. Biol. Rhythms* **15**, 135-154.
- HELFRICH-FÖRSTER, C. (2001) The locomotor activity rhythm of *Drosophila melanogaster* is controlled by a dual oscillator system. *J. Insect Physiol.* **47**, 877-887.
- HELFRICH-FÖRSTER, C. and HOMBERG, U. (1993) Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. *J. Comp. Neurol.* **337**, 177-190.
- HELFRICH-FÖRSTER, C., STENGL, M. and HOMBERG, U. (1998) Organization of the circadian system in insects. *Chronobiol. Intern.* **15**, 567-594.
- HELFRICH-FÖRSTER, C., TÄUBER, M., PARK, J.H., MÜHLIG-VERSEN, M., SCHNEUWLY, S. and HOFBAUER, A. (2000) Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci.* **20**, 3339-3353.
- HELFRICH-FÖRSTER, C., WINTER, C., HOFBAUER, A., HALL, J.C. and STANEWSKY, R. (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30**, 249-261.
- HELLER, H. C. and POULSON, T. L. (1970) Circannian rhythms-II. Endogenous and exogenous factors controlling reproduction and hibernation in chipmunks (*Eutamias*) and ground squirrels (*Spermophilus*). *Comp. Biochem. Physiol.* **33**, 357-383.
- HEMPPEL, G. and HEMPEL, I. (1959) Über die tagliche verteilung der Laufaktivität bei Käfern des hohen nordens. *Naturwiss.* **42**, 77-88.
- HENDRICKS, J.C., FINN, S.M., PANCKERI, K.A., CHAVKIN, J., WILLIAMS, J.A., SEHGAL, A. and PACK, A.L. (2000) Rest in *Drosophila* is a sleep-like state. *Neuron* **25**, 129-138.
- HENRICH, V.C. and DENLINGER, D.L. (1982) A maternal effect that eliminates pupal diapause in progeny of the flesh fly, *Sarcophaga bullata*. *J. Insect Physiol.* **28**, 881-884.
- HENRICH, V.C. and DENLINGER, D.L. (1983) Genetic differences in pupal diapause incidence between two selected strains of the flesh fly. *J. Heredity* **74**, 371-374.
- HEPBURN, H.R. (1985) Structure of the integument. In: Kerkut, G.A. and Gilbert, L.I. (Eds.) *Comprehensive Insect Physiology Biochemistry and Pharmacology*, vol 3., Pergamon Press, Oxford, pp. 1-58.
- HERAN, H. (1962) Anemotaxis und Fluchtorientierung des Bachlaufers *Velia caprai* Tam. (= *V. currens* F.). *Z. vergl. Physiol.* **46**, 129-149.
- HERZOG, E.D. and TOSINI, G. (2001) The mammalian circadian clock shop. *Seminars in Cell and Devel. Biol.* **12**, 295-303.
- HESTERLEE, S. and MORTON, D.B. (1996) Insect physiology: the emerging story of ecdysis. *Current Biology* **6**, 648-650.
- HEWES, R.S. and TRUMAN, J. W. (1991) The roles of central and peripheral eclosion hormone release in the control of ecdysis behaviour in *Manduca sexta*. *J. Comp. Physiol. A* **168**, 697-707.

- HIDAKA, T. and HIRAI, Y. (1970) Effect of non-24-hour photoperiod and light interruption of the dark phase on diapause determination in *Papilio xuthus* L. *Proc. Jap. Acad.* **46**, 541-545.
- HIGHKIN, H. R. and HANSON, L. B. (1954) Possible interaction between light-dark cycles and endogenous daily rhythms on the growth of tomato plants. *Plant Physiol.* **29**, 301-302.
- HIGHNAM, K. C. (1958) Activity of the brain/corpora cardiaca system during pupal diapause 'break' in *Mimastiliae* (Lepidoptera). *Q. Jl microsc. Sci.* **99**, 73-88.
- HIGUCHI, C. and KIMURA, M.T. (1985) influence of photoperiod on the low temperature acclimation for cold-hardiness in *Drosophila auraria*. *Physiol. Entomol.* **10**, 303-308.
- HILLMAN, W. S. (1956) Injury of tomato plants by continuous light and unfavorable photoperiodic cycles. *Am. J. Bot.* **43**, 89-96.
- HILLMAN, W. S. (1964) Endogenous circadian rhythms and the response of *Lemma perpusilla* to skeleton photoperiods. *Am. Nat.* **98**, 323-328.
- HILLMAN, W. S. (1973) Non-circadian photoperiodic timing in the aphid *Megoura*. *Nature, Lond.* **242**, 128-129.
- HINKS, C. F. (1967) Relationship between serotonin and the circadian rhythm in some nocturnal moths. *Nature, Lond.* **214**, 386-387.
- HINTERMANN, E., GRIEDER, N.C., AMHERD, R., BRODBECK, D. and MEYER, U.A. (1996) Cloning of an arylalkylamine *N*-acetyltransferase (aaNAT1) from *Drosophila melanogaster* expressed in the nervous system and the gut. *Proc. Nat. Acad. Sci. US* **93**, 12315-12320.
- HINTON, H. E. (1951) A new chironomid from Africa, the larva of which can be dehydrated without injury. *Proc. zool. Soc. Lond.* **121**, 371-380.
- HINTON, H. E. (1960) Cryptobiosis in the larva of *Polypedilum vanderplanki* Hint. (Chironomidae). *J. Insect Physiol.* **5**, 286-300.
- HODEK, I. (1960) Hibernation-bionomics in Coccinellidae. *Acta Soc. ent. Cechoslov.* **57**, 1-20.
- HODEK, I. (1962) Experimental influencing of the imaginal diapause in *Coccinella septempunctata* L. (Col., Coccinellidae), 2nd. part. *Acta Soc. ent. Cechoslov.* **59**, 297-313.
- HODEK, I. (1967) Bionomics and ecology of predaceous Coccinellidae. *A. Rev. Ent.* **12**, 79-104.
- HODEK, I. (1968) Diapause in females of *Pyrrhocoris apterus* L. (Heteroptera). *Acta ent. Bohemoslov.* **65**, 422-435.
- HODEK, I. and ČERKASOV, J. (1958) A study of the imaginal diapause of *Semiadalia undecimnotata* Schneid. (Coccinellidae, Col.) in the open. I. *Acta Soc. zool. Bohemoslov.* **22**, 180-192.
- HODEK, I. and ČERKASOV, J. (1960) Prevention and artificial induction of the imaginal diapause in *Coccinella 7-punctata* L. *Nature, Lond.* **187**, 345.
- HODGETTS, R.B. and KONOPKA, R.J. (1973) Tyrosine and catecholamine metabolism in wild-type *Drosophila melanogaster* and a mutant, *ebony*. *J. Insect Physiol.* **19**, 1211-1220.
- HODKOVA, M. (1976) Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. *Nature, Lond.* **263**, 521-523.
- HODKOVA, M. (1977) Functions of the neuroendocrine complex in diapausing *Pyrrhocoris apterus* females. *J. Insect Physiol.* **23**, 23-28.
- HODKOVA, M. (1989) Indication of the role of melatonin in the regulation of reproduction in *Pyrrhocoris apterus* (Heteroptera). *Acta Entomol. Bohemoslov.* **86**, 81-85.
- HODKOVA, M. (1994) Photoperiodic regulation of mating behaviour in the linden bug, *Pyrrhocoris apterus*, is mediated by a brain inhibiting factor. *Experientia* **50**, 742-744.
- HODKOVA, M. and HODEK, I. (1987) Photoperiodic summation is temperature-dependent in *Pyrrhocoris apterus* (L.). *Experientia* **43**, 454-456.
- HODKOVA, M., ZIEGLEROVA, J. and HODEK, I. (1991) Diapause in males of *Pyrrhocoris apterus* (L.) (Heteroptera) and its dependence on photoperiod and the activity of females. *Zool. Jb. Syst.* **118**, 279-285.
- HOELSCHER, C. E. and VINSON, S. B. (1971) The sex ratio of a hymenopterous parasitoid, *Campoletis perdinctus*, as affected by photoperiod, mating, and temperature. *Ann. ent. Soc. Am.* **64**, 1373-1376.
- HOFBAUER, A. and BUCHNER, E. (1989) Does *Drosophila* have seven eyes? *Naturwissenschaften* **76**, 335-336.
- HOFFMANN, H.J. (1970) Neuro-endocrine control of diapause and oocyte maturation in the beetle, *Pterostichus nigrita*. *J. Insect Physiol.* **16**, 629-642.
- HOFFMAN, J.A. and LAGEAU, M. (1985) Endocrine aspects of embryonic development in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 1. Pergamon Press, Oxford, pp. 435-460.
- HOFFMANN, K. (1953) Experimentelle Änderung des Richtungsfinden beim Star durch Beeinflussung der 'inneren Uhr'. *Naturwissenschaften* **40**, 608-609.

- HOFFMANN, K. (1954) Versuche zu der Richtungsfinden der Vögel enthaltenen Zeitschätzung. *Z. Tierpsychol.* **11**, 453-475.
- HOFFMANN, K. (1960) Experimental manipulation of the orientational clock in birds. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 379-387.
- HOFFMANN, K. (1965) Overt circadian frequencies and circadian rule. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 87-94. North-Holland, Amsterdam.
- HOFFMANN, K. (1969) Circadiane Periodik bei Tupajas (*Tupaia glis*) in Konstanten Bedingungen *Zool. Anz. Suppl.* **33**, 171-177.
- HOFFMANN, K. (1971) Biological clocks in animal orientation and in other functions. *Proc. Int. Symp. Circadian Rhythmicity (Wageningen, 1971)*, pp. 175-205.
- HOFFMANN, K. (1971) Splitting of the circadian rhythm as a function of light intensity. In: *Biocronometry* (Ed. MENAKER, M.), pp. 134-150. National Academy of Sciences, Washington, D.C.
- HOFFMAN, K.H. (1995) Oogenesis and the female reproductive system. In: Leather, S.R. and Hardie, J. (Eds.), *Insect Reproduction*, CRC Press, London, pp. 1-32.
- HOFFMANN, R. J. (1973) Environmental control of seasonal variation in the butterfly, *Colias eurytheme*. I. Adaptive aspects of a photoperiodic response. *Evolution*, **27**, 387-397.
- HOFFMANN, R. J. (1974) Environmental control of seasonal variation in the butterfly *Colias eurytheme*: effects of photoperiod and temperature on pteridine pigmentation. *J. Insect Physiol.* **20**, 1913-1924.
- HOFFMANN, R. J. (1978) Environmental uncertainty and evolution of physiological adaptation in *Colias* butterflies. *Am. Nat.* **112**, 999-1015.
- HOFFMANN, K., GUNDEROTH-PALMOWSKI, M. and ENGELMANN, W. (1978) Further evidence for period lengthening effect of Li+ on circadian rhythms. *Z. Naturforsch. C*, **33**, 231-234.
- HOLLINGSWORTH, M. J. (1969) Fluctuating temperatures and the length of life in *Drosophila*. *Nature, Lond.* **221**, 857-858.
- HOMBERG, U., DAVIS, N.T. and HILDEBRAND, J.G. (1991) Peptide immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. *J. Comp. Neurology* **303**, 35-52.
- HOMBERG, U., WÜRDEN, S., DIRCKSEN, H. and RAO, K.R. (1991) Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. *Cell Tissue Res.* **266**, 343-357.
- HONEK, A. (1972) Selection for non-diapause in *Aelia acuminata* and *A. rostrata* (Heteroptera, Pentatomidae) under various selective pressures. *Acta Entomol. Bohemoslov.* **69**, 73-77.
- HONG, S.-F. and SAUNDERS, D.S. (1994) Effects of constant light on the rhythm of adult locomotor activity in the blow fly, *Calliphora vicina*. *Physiol. Entomol.* **19**, 319-324.
- HONG, S.-F. and SAUNDERS, D.S. (1998) Internal desynchronisation in blow fly (*Calliphora vicina*) locomotor activity rhythms: evidence for a complex circadian pacemaker. *Biol. Rhythm Res.* **29**, 387-396.
- HORODYSKI, F.M. (1996) Neuroendocrine control of insect ecdysis by eclosion hormone. *J. Insect Physiol.* **42**, 917-924.
- HORODYSKI, F.M., EWER, J., RIDDIFORD, L.M. and TRUMAN, J.W. (1993). Isolation, characterization and expression of the eclosion hormone gene of *Drosophila melanogaster*. *Eur. J. Biochem.* **215**, 221-228.
- HORWATH, K.L. and DUMAN, J.G. (1982) Involvement of the circadian system in photoperiodic regulation of insect antifreeze proteins. *J. exp. Zool.* **219**, 269-270.
- HORWATH, K.L. and DUMAN, J.G. (1983) Photoperiodic and thermal regulation of antifreeze protein levels in the beetle *Dendroides canadensis*. *J. Insect Physiol.* **29**, 907-917.
- HORWATH, K.L. and DUMAN, J.G. (1984) Further studies on the involvement of the circadian system in photoperiodic control of antifreeze protein production in the beetle *Dendroides canadensis*. *J. Insect Physiol.* **30**, 947-955.
- HOUSE, H. L. (1967) The decreasing occurrence of diapause in the fly *Pseudosarcophaga affinis* through laboratory-reared generations. *Can. J. Zool.* **45**, 149-153.
- HOY, M.A. (1977) Rapid response to selection for a nondiapausing gypsy moth. *Science* **196**, 1462-1463.
- HUANG, Z.J., CURTIN, K.D. and ROSBASH, M. (1995) PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. *Science* **267**, 1169-1172.
- HUGHES, R. D. (1960) Induction of diapause in *Erioischia brassicae* Bouché (Dipt., Anthomyiidae). *J. exp. Biol.* **37**, 218-223.
- HUIGNARD, J. (1983) Transfer and fate of male secretions deposited in the spermatophore of females of *Acanthoscelides obtectus* Say. *J. Insect Physiol.* **29**, 55-63.
- HUNT, R.E. and HAYNES, K.F. (1990) Periodicity in the quantity and blend ratios of pheromone components in

- glands and volatile emissions of mutant and normal cabbage looper moths, *Trichoplusia ni*. *J. Insect Physiol.* **36**, 769-774.
- HUNTER-ENSOR, M., OUSLEY, A. and SEHGAL, A. (1996) Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell* **84**, 677-685.
- ICHIKAWA, M. and NISHITSUTSUJI-UWO, J. (1955) *Mem. Coll. Sci. Kyoto B*, **22**, 11-15.
- ICHIKAWA, T. (1998) Activity patterns of neurosecretory cells releasing pheromonotropic neuropeptides in the moth *Bombyx mori*. *Proc. Nat. Acad. Sci., USA* **95**, 4055-4060.
- ICHIKAWA, T., HASEGAWA, K., SHIMIZU, I., KATSUNO, K., KATAOKA, H. and SUZUKI, A. (1995) Structure of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the *Bombyx mori*. *Zool. Sci.* **12**, 703-712.
- ICHIKAWA, T. and ITO, K. (1999) Calling behaviour modulates heartbeat reversal rhythm in the silkworm *Bombyx mori*. *Zool. Sci.* **16**, 203-209.
- ICHIKAWA, T. and KAZUMI, I. (1999) Calling behaviour modulates heartbeat reversal rhythm in the silkworm *Bombyx mori*. *Zool. Sci.* **16**, 203-209.
- IGLESIAS, F., JACQUIN-JOLY, E., MARCO, M.-P., CAMPS, F. and FABRIUS, G. (1999) Temporal distribution of PBAN-like immunoreactivity in the haemolymph of *Mamestra brassicae* females in relation to sex pheromone production and calling behaviour. *Arch. Insect Biochem. Physiol.* **40**, 80-87.
- IMAI, K., KONNO, T., NAKAZAWA, Y., KOMIYA, T., ISOBE, M., KOGA, K., GOTO, T., YAGINUMA, T., SAKAKIBARA, K., HASEGAWA, K. and YAMASHITA, O. (1991) Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **39**, 889-895.
- INGRAM, B. R. (1976) Effects of photoperiod and temperature on abnormal wing-pad development in two species of Odonata. *Can. J. Zool.* **54**, 1103-1110.
- ISHIZAKI, H. and SUZUKI, A. (1994) The brain secretory peptides that control moulting and metamorphosis of the silkworm, *Bombyx mori*. *Intern. J. Develop. Biology* **38**, 301-310.
- ISTOCK, C.A., ZISFEIN, J. and VAVRA, K.J. (1976) Ecology and evolution of the pitcher plant mosquito. 2. The substructure of fitness. *Evolution* **30**, 535-547.
- ITAGAKI, H. and CONNER, W.E. (1988) Calling behaviour of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Ent. Soc. Am.* **81**, 795-807.
- ITOH, M.T., HATTORI, A., SUMI, Y. and SUZUKI, T. (1994) Identification of melatonin in different organs of the cricket, *Gryllus bimaculatus*. *Zool. Sci.* **11**, 577-581.
- ITOH, M.T., HATTORI, A., SUMI, Y. and SUZUKI, T. (1995) Day-night changes in melatonin levels in different organs of the cricket (*Gryllus bimaculatus*). *J. Pineal Res.* **18**, 165-169.
- ITOH, M.T., NOMURA, T. and SUMI, Y. (1997) Hydroxyindole-O-methyltransferase activity in the silkworm (*Bombyx mori*). *Brain Res.* **765**, 61-66.
- ITOH, M.T. and SUMI, Y. (1998a) Melatonin and serotonin N-acetyltransferase activity in developing eggs of the cricket *Gryllus bimaculatus*. *Brain Research* **781**, 91-99.
- ITOH, M.T. and SUMI, Y. (1998b) Circadian clock controlling arylalkylamine N-acetyltransferase-like activity in the cricket (*Gryllus bimaculatus*) egg. *Brain Res.* **799**, 172-175.
- ITOH, M.T. and SUMI, Y. (2000) Circadian clock controllin egg hatching in the cricket (*Gryllus bimaculatus*) *J. Biol. Rhythms* **15**, 241-245.
- IZQUIERDO, J.I. (1991) How does *Drosophila melanogaster* overwinter? *Entomol. Exp. Appl.* **59**, 51-58.
- JACKLET, J. W. (1969) Circadian rhythm of optic nerve impulses recorded in darkness from isolated eye of *Aplysia*. *Science, Wash.* **164**, 562-563.
- JACKLET, J. W. (1971) A circadian rhythm in optic nerve impulses from an isolated eye in darkness. In *Biochronometry* (Ed. MENAKER, M.), pp. 351-362. National Academy of Sciences, Washington.
- JACKSON, F.R. (1983) The isolation of biological rhythm mutations on the autosomes of *Drosophila melanogaster*. *J. Neurogenet.* **1**, 3-15.
- JACKSON, F.R., SCHROEDER, A.J., ROBERTS, M.A., McNEIL, G.P., KUME, K. and AKTEN, B. (2001) Cellular and molecular mechanisms of circadian control in insects. *J. Insect Physiol.* **47**, 833-842.
- JACQUIN-JOLY, E., BURNET, M., FRANCOIS, M.C., AMMAR, D., NAGNAN LeMEILLIOL, P. and DESCOINS, C. (1998) cDNA cloning and sequence determination of the pheromone biosynthesis activation neuropeptide of *Mamestra brassicae*: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* **28**, 251-258.
- JANDER, R. (1957) *Z. vergl. Physiol.* **40**, 162-238.
- JEGLA, T. C. and POULSON, T. L. (1970) Circannian rhythms-I. Reproduction in the cave crayfish, *Orconectes pellucidus inermis*. *Comp. Biochem. Physiol.* **33**, 347-355.

- JENNER, C. E. and ENGELS, W. L. (1952) The significance of the dark period in the photoperiodic response of male juncos and white-throated sparrows. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **103**, 345-355.
- JEWETT, M.E., FORGER, D.B. and KRONAUER, R.E. (1999) Revised limit cycle oscillator model of human circadian pacemaker. *J. Biol. Rhythms* **14**, 493-499.
- JEWETT, M.E. and KRONAUER, R.E. (1998) Refinement of a limit cycle oscillator model of the effects of light on the human circadian pacemaker. *J. theor. Biol.* **192**, 455-465.
- JOHNSON, C.H. (1992) Phase response curves: What can they tell us about circadian clocks? In *Circadian Clocks from Cell to Human*, eds. HIROSHIGE, T. and HONMA, K. Hokkaido Univ. Press, Sapporo. Pp. 209-249.
- JOHNSON, C.H., KNIGHT, M., TREWAWAS, A. and KONDO, T. (1998) A clockwork green: circadian programs in photosynthetic organisms. In *Biological Rhythms and Photoperiodism in Plants*, ed. LUMSDEN, P.J. and MILLAR, A.J. Bios Scientific Publishers Ltd., Oxford. Pp. 1-34.
- JOHNSON, A. and KARLSSON, H. G. (1972a) A feedback model for biological rhythms. I - Mathematical description and basic properties of the model. *J. theoret. Biol.* **36**, 153-174.
- JOHNSON, A. and KARLSSON, H. G. (1972b) The *Drosophila* eclosion rhythm, the transformation method, and the fixed point theorem. Department of Electrical Measurements, Lund Institute of Technology, Report No. 2/1972, November 15, 1972.
- JOHNSTON, J.S. and ELLISON, J.R. (1982) Exact age determination in laboratory and field-caught *Drosophila*. *J. Insect Physiol.* **28**, 773-779.
- JOLY, P. (1945) La fonction ovarienne et son control humoral chez les Dytiscides. *Archs Zool. exp. gen.* **84**, 49-164.
- JONES, M. D. R. (1964) The automatic recording of mosquito activity. *J. Insect Physiol.* **10**, 343-351.
- JONES, M. D. R. (1973) Delayed effect of light on the mosquito 'clock'. *Nature, Lond.* **245**, 384-385.
- JONES, M. D. R. (1976) Persistence in continuous light of a circadian rhythm in the mosquito *Culex pipiens fatigans* Wied. *Nature, Lond.* **261**, 491-492.
- JONES, M.D.R. (1982) Coupled oscillators controlling circadian flight activity in the mosquito, *Culex pipiens quinquefasciatus*. *Physiol. Entomol.* **7**, 281-289.
- JONES, M. D. R., CUBBIN, C. M. and MARSH, D. (1972a) The circadian rhythm of flight activity of the mosquito *Anopheles gambiae*: the light-on response. *J. exp. Biol.* **57**, 337-346.
- JONES, M. D. R., CUBBIN, C. M. and MARSH, D. (1972b) Light-on effects and the question of bimodality in the circadian flight activity of the mosquito *Anopheles gambiae*. *J. exp. Biol.* **57**, 347-357.
- JONES, M. D. R., FORD, M. G. and GILLET, J. D. (1966) Light-on and light-off effects on the circadian flight activity in the mosquito *Anopheles gambiae*. *Nature, Lond.* **211**, 871-872.
- JONES, M. D. R. and GUBBINS, S. J. (1977) Modification of circadian flight activity in the mosquito *Anopheles gambiae* after insemination. *Nature, Lond.* **268**, 731-732.
- JONES, M. D. R. and GUBBINS, S. J. (1978) Changes in the circadian flight activity of the mosquito *Anopheles gambiae* in relation to insemination, feeding and oviposition. *Physiol. Ent.* **3**, 213-220.
- JONES, M. D. R., HILL, M. and HOPE, A. M. (1967) The circadian flight activity of the mosquito *Anopheles gambiae*: phase setting by the light regime. *J. exp. Biol.* **47**, 503-511.
- JONES, M. D. R. and REITER, R. (1975) Entrainment of the pupation and adult activity rhythms during development in the mosquito *Anopheles gambiae*. *Nature, Lond.* **254**, 242-244.
- JORDAN, R. G. and BRADSHAW, W. E. (1978) Geographic variation in the photoperiodic response of the western tree-hole mosquito *Aedes sierrensis*. *Ann. ent. Soc. Am.* **71**, 487-490.
- JOSHI, D.S. (1996) Psi-mutation affects phase angle difference, free-running period and phase shifts in *Aedes krombeini* (Stegomyia). *Biol. Rhythm Res.* **27**, 421-430.
- JOSHI, D.S. (1999) Latitudinal variation in locomotor activity rhythm in adult *Drosophila ananassae*. *Can. J. Zool.* **77**, 865-870.
- JURENKA, R.A., JACQUIN, E. and ROELOFS, W.L. (1991) Stimulation of pheromone biosynthesis in the moth *Helicoverpa zea*: action of a brain hormone on pheromone glands involves C^2 and cAMP as second messengers. *Proc. Nat. Acad. Sci., USA* **88**, 8621-8625.
- KAISER, W. (1988) Busy bees need rest, too. *J. comp. Physiol. A* **163**, 565-584.
- KAISER, W. and STEINER-KAISER, J. (1983) Neuronal correlates of sleep, wakefulness and arousal in a diurnal insect. *Nature* **301**, 707-709.
- KAJIURA, Z., NAKAGAKI, M. and TAKEI, R. (1993) Spermiogenesis of the testes of juvenile hormone-treated silkworm larvae, *Bombyx mori*. *Comp. Biochem. Physiol. A*, **106**, 495-499.

- KALOGIANNI, E. and THEOPHILIDES, G. (1993) Centrally generated rhythmic activity and modulatory function of the oviductal dorsal unpaired median (DUM) neurons in 2 orthopteran species (*Calliptamus* sp and *Decticus albifrons*). *J. Exp. Biology* **174**, 123-138.
- KALMUS, H. (1934) Über die Natur des Zeitgedächtnisses der Bienen. *Z. vergl. Physiol.* **20**, 405-419.
- KALMUS, H. (1935) Periodizität und Autochronie (Ideochronie) als Zeitregelnde Eigenschaften der Organismen. *Biologia generales*, **11**, 93-114.
- KALMUS, H. (1938) Die Lage des Aufnahmeorgans für die Schlupfperiodik von *Drosophila*. *Z. vergl. Physiol.* **26**, 362-365.
- KALMUS, H. (1940) Diurnal rhythms in the Axolotl larva and in *Drosophila*. *Nature, Lond.* **145**, 72-73.
- KALMUS, H. (1956) Sun navigation of *Apis mellifica* L. in the southern hemisphere. *J. exp. Biol.* **33**, 554-565.
- KALMUS, H. and WIGGLESWORTH, L.A. (1960) Shock excited systems as models for biological rhythms. *Cold Spring Harbor Symp. Quant. Biol.* **25**, 211-216.
- KAMIMURA, M. and TATSUKI, S. (1993) Diel rhythms of calling behaviour and pheromone, production of Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Chemical Ecol.* **19**, 2953-2963.
- KAMIMURA, M. and TATSUKI, S. (1994) Effects of photoperiodic changes on calling behaviour and pheromone production in the Oriental tobacco budworm moth, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* **40**, 731-734.
- KAMM, J. A. (1970) Effects of photoperiod and temperature on *Crambus trisectus* and *C. leachellus* cypridalis (Lepidoptera: Crambidae). *Ann. ent. Soc. Am.* **63**, 412-416.
- KAMM, J. A. (1972) Photoperiodic regulation of growth in an insect: response to progressive changes in daylength. *J. Insect Physiol.* **18**, 1745-1749.
- KAMM, J. A. (1972) Environmental influence on reproduction, diapause, and morph determination of *Anaphothrips obscurus* (Thysanoptera: Thripidae). *Environ. Ent.* **1**, 1-16.
- KANEKO, J. (1986) Effect of temperature on the timing of calling of the yellow peach moth, *Congethes punctiferalis* (Guenee) (Lepidoptera: Pyralidae). *Jap. J. Appl. ent. Zool.* **30**, 239-246.
- KANEKO, M., HAMBLEN, M.J. and HALL, J.C. (2000a) Involvement of the *period* gene in developmental time-memory: effect of the *per^{Short}* mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. *J. Biol. Rhythms* **15**, 13-30.
- KAPLANIS, J.N., TABOR, L.A., THOMPSON, M.J., ROBBINS, W.E. and SHORTINO, T.J. (1966) Assay for ecdysone (moulting hormone) activity using the housefly, *Musca domestica* L. *Steroids* **8**, 625-631.
- KASAI, M. and CHIBA, Y. (1987) Effects of optic lobe ablation on circadian activity in the mosquito, *Culex pipiens pallens*. *Physiol. Entomol.* **12**, 59-65.
- KEELEY, L. L. (1970) Diapause metabolism and rearing methods for the whitelined sphinx, *Celerio lineata* (Lepidoptera, Sphingidae). *Ann. ent. Soc. Am.* **63**, 905-907.
- KENNY, N.A.P., RICHARD, D.S., BRADLEY, H.K. and SAUNDERS, D.S. (1992) Photoperiodic sensitivity and diapause induction during ovarian, embryonic and larval development of the flesh fly, *sarcophaga argyrostoma*. *J. Biosciences* **17**, 241-251.
- KENNY, N.A.P. and SAUNDERS, D.S. (1991) Adult locomotor rhythmicity as "hands" of the maternal photoperiodic clock regulating larval diapause in the blowfly, *Calliphora vicina*. *J. Biol. Rhythms* **6**, 217-233.
- KERFOOT, W. B. (1967) The lunar periodicity of *Specodogastra texana*, a nocturnal bee (Hymenoptera, Halictidae). *Anim. Behav.* **15**, 479-486.
- KEVAN, D. K. M. (1944) The bionomics of the neotropical cornstalk borer, *Diatraea lineolata* Wlk in Trinidad, B.W.I. *Bull. ent. Res.* **35**, 23-30.
- KHOO, S. G. (1968) Experimental studies on diapause in stoneflies. I. Nymphs of *Capnia bifrons* (Newman). *Proc. R. ent. Soc. Lond. (A)*, **43**, 40-48.
- KIDOKORO, T. and MASAKI, S. (1978) Photoperiodic response in relation to variable voltinism in the ground cricket *Pteronemobius fascipes* Walker (Orthoptera: Gryllidae). *Jap. J. Ecol.* **28**, 291-298.
- KIKAKAWA, S. and CHIPPENDALE, G.M. (1983) Seasonal adaptations of populations of the southwestern corn borer, *Diatraea grandiosella*, from tropical and temperate regions. *J. Insect Physiol.* **29**, 561-567.
- KIMURA, M.T. (1982) Effects of photoperiod and temperature on reproductive diapause in *Drosophila testacea*. *Experientia* **38**, 371-372.
- KIMURA, M.T. (1990) Quantitative response to photoperiod during reproductive diapause in the *Drosophila auraria* species-complex. *J. Insect Physiol.* **36**, 147-152.
- KIMURA, M.T. and YOSHIDA, T. (1995) A genetic analysis of photoperiodic reproductive diapause in *Drosophila triauraria*. *Physiol. Entomol.* **20**, 253-256.

- KIMURA, T. and MASAKI, S. (1977) Brachypterism and seasonal adaptation in *Orgyia thyellina* Butler (Lepidoptera, Lymantriidae). *Kontyu*, **45**, 97-106.
- KIMURA, Y. and MASAKI, S. (1993) Hourglass and oscillator expression of photoperiodic diapause response in the cabbage moth *Mamestra brassicae*. *Physiol. Entomol.* **18**, 240-246.
- KIND, T. V. (1965) Neurosecretion and vultinism in *Orgyia antiqua* L. (Lepidoptera, Lymanthriidae). *Ent. Obozr.* **44**, 534-536. (In Russian.)
- KING, A. B. S. (1974) Photoperiodic induction and inheritance of diapause in *Pionea forficalis* (Lepidoptera: Pyralidae). *Ent. exp. & appl.* **17**, 397-409.
- KING, P. E. (1963) The rate of egg resorption in *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) deprived of hosts. *Proc. R. ent. Soc. Lond. (A)*, **38**, 98-100.
- KING, V.M. (1988) Advances in the model for the circadian organisation of the weta (*Hemideina thoracica*). Unpublished Masters thesis, University of Auckland.
- KINGAN, T.G. and ADAMS, M.E. (2000) Ecdysteroids regulate secretory competence in Inka cells. *J. exp. Biol.* **203**, 3011-3018.
- KINGAN, T.G., GRAY, W., ZITNAN, D. and ADAMS, M.E. (1997) Regulation of ecdysis-triggering hormone secretion by eclosion hormone. *J. Exp. Biol.* **200**, 3245-3256.
- KINGAN, T.G., RAINA, A.K., BLACKBURN, M. and MA, M. (1990) Distribution of PBAN-like immunoreactivity in the CNS of the corn earworm, *Heliothis zea*. *Neuroscience Abstr.* **16**, 856.
- KIRKPATRICK, C. M. and LEOPOLD, A. C. (1952) The role of darkness in sexual activity of the quail. *Science, Wash.* **116**, 280-281.
- KISIMOTO, R. (1956) Effect of diapause in the fourth larval instar on the determination of wing form in the adult of the small brown plant hopper, *Delphacodes striatella* Fallen. *Oyo-Kontyu*, **12**, 202-210.
- KISIMOTO, R. (1959) Studies on the diapause in the planthoppers and leafhoppers. III. Sensitivity of various larval stages to photoperiod and the forms of ensuing adults in the green rice leafhopper, *Nephotettix cincticeps*. *Japan. J. appl. Ent. Zool.* **3**, 200-207.
- KITAMURA, A., NAGASAWA, H., KATAOKA, H., INOUE, T., MATSUMOTO, S., ANDO, T. and SUZUKI, A. (1989) Amino acid sequence of pheromone biosynthesis-activating-neuropeptide (PBAN) of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Comm.* **163**, 520-526.
- KLARSFELD, A. and ROUYER, F. (1998) Effects of circadian mutations and LD periodicity on the life span of *Drosophila melanogaster*. *J. Biol. Rhythms* **13**, 471-478.
- KLEBER, E. (1935) Hat das Zeitgedächtnis der Bienen biologische Bedeutung? *Z. vergl. Physiol.* **22**, 221-262.
- KLEMM, E. and NINNEMANN, H. (1976) Detailed action spectrum for the delay shift in pupal emergence of *Drosophila pseudoobscura*. *Photochem. Photobiol.* **24**, 369-371.
- KLOTTER, K. (1960) Theoretical analysis of some biological models. In *Biological Clocks. Cold Spring Harb. Symp. Quant. Biol.* **25**, 189-196.
- KLUG, H. (1958) Histo-physiologische Untersuchungen Über die Aktivitätsperiodik bei Carabiden. *Wiss. Z. Humboldt. Univ. Berlin, Math.-Naturw. Reihe* **8**, 405-434.
- KOCH, P.B. and BÜCKMANN, D. (1987) hormonal control of seasonal morphs by the timing of ecdysteroid release in *Araschnia levana* L. (Nymphalidae: Lepidoptera). *J. Insect Physiol.* **33**, 823-829.
- KOEHLER, W. and FLEISSNER, G. (1978) Internal desynchronization of bilaterally organised circadian oscillators in the visual system of insects. *Nature, Lond.* **274**, 708-710.
- KOEPPE, J.K., FUCHE, M., CHEN, T.T., HUNT, L.-M., KOVALICK, G.E. and BRIERS, T. (1985) The role of juvenile hormone in reproduction. In: Kerkut, G.A. and Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. **8**. Pergamon Press, Oxford, pp. 165-203.
- KOGURE, M. (1933) The influence of light and temperature on certain characters of the silk-worm, *Bombyx mori*. *J. Dept. Agr. Kyushu Univ.* **4**, 1-93.
- KOMAROVA, O. S. (1949) the conditions evoking diapause in the vine leafroller (*Polychrosis botrana* Schiff.). *Dokl. Akad. Nauk SSSR*, **68**, 789-792. (In Russian.)
- KONO, Y. (1970) Photoperiodic induction of diapause in *Pieris rapae crucivora* Boisduval (Lepidoptera: Pieridae). *Appl. Ent. Zool.* **5**, 213-224.
- KONO, Y. (1973) Light and electron microscope studies on the neurosecretory control of diapause incidence in *Pieris rapae crucivora*. *J. Insect Physiol.* **19**, 255-272.
- KONOPKA, R.J. (1979) Genetic dissection of the *Drosophila* circadian system. *Federation Proc.* **38**, 2602-2605.
- KONOPKA, R.J. (1987) Neurogenetics of *Drosophila* circadian rhythms. In *Evolutionary Genetics of Invertebrate Behavior*. Huettel, M.D., ed. Plenum, New York, pp. 215-221.

- KONOPKA, R.J. (1988) A variegating long-period clock mutant of *Drosophila melanogaster*. *Life Sci. Adv. (Genet.)* **7**, 39-41.
- KONOPKA, R. and BENZER, S. (1971) Clock mutants of *Drosophila melanogaster*. *Proc. Natn. Acad. Sci. U.S.A.* **68**, 2112-2116.
- KONOPKA, R.J., HAMBLEN-COYLE, M.J., JAMISON, C.F. and HALL, J.C. (1994) An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. *J. Biol. Rhythms* **9**, 189-216.
- KONOPKA, R.J., KYRIACOU, C.P. and HALL, J.C. (1996) Mosaic analysis in the *Drosophila* CNS of circadian and courtship-song rhythms affected by a *period* clock mutation. *J. Neurogenet.* **11**, 117-139.
- KONOPKA, R.J. and ORR, D. (1980) Effects of a clock mutation on the subjective day - implications for a membrane model of the *Drosophila* circadian clock. In *Development and Neurobiology of Drosophila*. Siddiqi, O., Babu, P., Hall, L.M. and Hall, J.C., eds. Plenum, New York, pp. 409-415.
- KONOPKA, R. J., PITTENDRIGH, C.S. and ORR, D. (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *J. Neurogenetics* **6**, 1-10.
- KONOPKA, R. and WELLS, S. (1981). Unpublished observations.
- KONOPKA, R.J. and WELLS, S. (1980) *Drosophila* clock mutations affect the morphology of a brain neurosecretory cell group. *J. Neurobiol.* **11**, 411-415.
- KOOLMAN, J. (Ed.). (1989) *Ecdysone*, Georg Thieme Verlag, Stuttgart.
- KOOLMAN, J. (1990) Ecdysteroids. *Zoological Science* **7**, 563-580.
- KÖPPÄ, P. (1970) Studies on the thrips (Thysanoptera) species most commonly occurring on on cereals in Finland. *Ann. Agric.fenn.* **9**, 191-265.
- KOU, R. (1992) Calling behaviour and pheromone titre in the smaller tea tortrix moth, *Adoxophyes* spp. (Lepidoptera: Tortricidae). *J. Chem. Ecology* **18**, 855-861.
- KOU, R. and CHOW, Y.S. (1987) Calling behaviour of the cotton bollworm, *Heliothis armigera* (Lepidoptera: Noctuidae). *Ann. Ent. Soc. Am.* **80**, 490-493.
- KOUDELE, K., STOUT, J.F. and REICHERT, D. (1987) Factors which influence female crickets (*Acheta domesticus*) phonotactic and sexual responsiveness to males. *Physiol. Entomol.* **12**, 67-80.
- KOVEOS, D.S. and VEERMAN, A. (1994) Accumulation of photoperiodic information during diapause development in the spider mite *Tetranychus urticae*. *J. Insect Physiol.* **40**, 701-707.
- KOVEOS, D.S., KROON, A. and VEERMAN, A. (1993) Geographic variation of diapause intensity in the spider mite *Tetranychus urticae*. *Physiol. Entomol.* **18**, 50-56.
- KRAMER, G. (1950) Weitere Analyse der Faktoren, welche die Zugaktivität des gekäfigten Vogels orientieren. *Naturwissenschaften*, **37**, 377-378.
- KRAMER, K.J. and KOGA, D. (1986) Insect chitin. Physical state, degradation and metabolic regulation. *Insect Biochem.* **16**, 851-877.
- KREHAN, I. (1970) Die Steuerung der Jahresrhythmik und Diapause bei Larval- und Imago-über-winteren der Gattung *Pterostichus* (Col., Carab.). *Oecologia (Berl.)*, **6**, 58-105.
- KRIGER, F.L. and DAVEY, K.G. (1984) Identified neurosecretory cells in the brain of female *Rhodnius prolixus* contain a myotropic peptide. *Can. J. Zool.* **52**, 1720-1729.
- KRISHNAN, B., DRYER, S.E. and HARDIN, P.E. (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* **400**, 375-378.
- KRISHNAN, B., LEVINE, J.D., LYNCH, K.S., DOWSE, H.B., FUNES, P., HALL, J.C., HARDIN, P.E. and DRYER, S.E. (2001) A novel role for cryptochrome in a *Drosophila* circadian oscillator. *Nature* **411**, 313-317.
- KRISTENSEN, B.I. (1966) Incorporation of tyrosine into the rubber-like cuticle of locusts studied by autoradiography. *J. Insect Physiol.* **12**, 173-177.
- KRONAUER, R.E., CZEISLER, C.A., PILATO, S.F., MOORE-EDE, M.C. and WEITZMAN, E.D. (1982) Mathematical model of the human circadian system with two interacting oscillators. *Am. J. Physiol.* **242** (Regulatory Integrative Comparative Physiology 11), R3-R17.
- KUBLI, E (1992) The sex peptide. *BioEssays* **14**, 779-784.
- KUTSCH, W. and HUBER, F. (1989) Neural basis of song production. In: Huber, F., Moore, T.E. and Loher, W. (Eds.), *Cricket Behaviour and Neurobiology*, Cornell University Press, Ithaca, pp. 262-309.
- KUWAHARA, Y., ADACHI, S. and TSUCHIDA, N. (1983) Bombykol content in female silkworm moth *Bombyx mori* (Lepidoptera: Bombycidae): Effect of age, mating and body weight. *App. Ent. Zool.* **18**, 182-190.
- KYORKU, C. and BRADY, J. (1994) A free-running bimodal circadian rhythm in the tsetse fly, *Glossina longipennis*. *J. Insect Physiol.* **40**, 63-67.

- KYRIACOU, C.P. and HALL, J.C. (1980) Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc. Nat. Acad. Sci. US* **77**, 6929-6933.
- KYRIACOU, C.P. and ROSATO, E. (2000). Squaring up the E-box. *J. Biol. Rhythms* **15**, 483-490.
- LaCHANCE, L.E., RICHARD, R.D. and RUUD, R.L. (1977) Movement of cupyrene sperm bundles from the testis and storage in the ductus ejaculatoris duplex of the male pink bollworm: Effects of age, strain, irradiation, and light. *Ann. Ent. Soc. Am.* **70**, 647-651.
- LAGEAUX, M., HETRU, C., GOLTZENE, E., KAPPLER, C. and HOFFMANN, J.A. (1979) Ecdysone titre and metabolism in relation to cuticulogenesis in embryos of *Locusta migratoria*. *J. Insect Physiol.* **25**, 709-723.
- LAKIN-THOMAS, P.L. (2000) Circadian Rhythms: New functions for old clock genes? *Trends in Genetics* **16**, 135-142.
- LALL, A.B. (1993) Nightly increase in visual sensitivity correlated with bioluminescent flashing activity in the firefly *Photuris versicolor* (Coleoptera: Lampyridae). *J. Exp. Zool.* **265**, 609-612.
- LAMPRECECHT, G. and WEBER, F. (1973) Mitnahme, Frequenzdemultiplikation und Maskierung der Laufaktivität von *Carabus*-Arten (Coleoptera) durch Lichtzyklen. *J. Insect Physiol.* **19**, 1579-1590.
- LAMPRECHT, G. and WEBER, F. (1977) Die Lichtempfindlichkeit der Circadianen rhythmik dreier Höhlenkäfer arten der Gattung *Laemostenus*. *J. Insect. Physiol.* **23**, 445-452.
- LANKINEN, P. (1986a) Geographical variation in circadian eclosion rhythms and photoperiodic adult diapause in *Drosophila littoralis*. *J. comp. Physiol. A* **159**, 123-142.
- LANKINEN, P. (1986b) Genetic correlation between circadian eclosion rhythm and photoperiodic diapause in *Drosophila littoralis*. *J. Biol. Rhythms* **1**, 101-118.
- LANKINEN, P. (1993a) North-south differences in circadian eclosion rhythm in European populations of *Drosophila subobscura*. *Heredity* **71**, 210-218.
- LANKINEN, P. (1993b) Characterization of *linne*, a new autosomal eclosion rhythm mutant in *Drosophila subobscura*. *Behav. Genet.* **23**, 359-367.
- LANKINEN, P. and RIIHIMAA, A.J. (1992) Weak circadian eclosion rhythmicity in *Chymomyza costata* (Diptera: Drosophilidae), and its independence of diapause type. *J. Insect Physiol.* **38**, 803-811.
- LAUDEHO, Y. LIAROPOULOS, C. and CANARD, M. (1978) Etude, pendant la periode automnale du rythme de sortie hors des fruits des larves du dernier age de la mouche de l'olive *Dacus oleae* (Gmel.) (Diptera, Trypetidae). *Ann. Zool. Ecol. anim.* **10**, 37-50.
- LAVIALLE, M. and DUMORTIER, B. (1990) metabolic correlates in the working of an insect putative photoperiodic clock. *J. comp. Physiol. A* **166**, 785-789.
- LEE, H.J. and WU, Y.L. (1994) Mating effects on the feeding and locomotion of the German cockroach, *Blattella germanica*. *Physiol. Entomol.* **19**, 39-45.
- LEE, H.T.Y. (1948) A comparative morphological study of prothoracic glandular bands of some lepidopteran larvae with special reference to their innervation. *Ann. Ent. Soc. Am.* **41**, 200-205.
- LEE, K.-Y. and DENLINGER, D.L. (1996) Diapause-regulated proteins in the gut of first instar larvae of the gypsy moth, *Lymantria dispar*, and the effects of KK-42 and neck ligation on expression. *J. Insect Physiol.* **42**, 423-431.
- LEE, K.-Y. and DENLINGER, D.L. (1997) A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* **43**, 289-296.
- LEE, R.E. Jr. and DENLINGER, D.L. (1991) *Insects at Low Temperature* (Eds.). Chapman and Hall, New York and London, 513 pp.
- LEES, A. D. (1953a) Experimental factors controlling the evocation and termination of diapause in the fruit tree red spider mite *Metatetranychus ulmi* Koch (Acarina: Tetranychidae). *Ann. appl. Biol.* **40**, 449-486.
- LEES, A. D. (1953b) The significance of the light and dark phases in the photoperiodic control of diapause in *Metatetranychus ulmi* Koch. *Ann. appl. Biol.* **40**, 487-497.
- LEES, A. D. (1955) *The Physiology of Diapause in Arthropods*. Cambridge University Press.
- LEES, A. D. (1959) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton - I. The influence of these factors on apterous virginoparae and their progeny. *J. Insect Physiol.* **3**, 92-117.
- LEES, A. D. (1960a) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton - II. The operation of the 'interval timer' in young clones. *J. Insect Physiol.* **4**, 154-175.
- LEES, A. D. (1960b) Some aspects of animal photoperiodism. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 261-268.
- LEES, A. D. (1961) Clonal polymorphism in aphids. *Symp. R. ent. Soc. Lond.* **1**, 68-79.

- LEES, A. D. (1963) The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton - III. Further properties of the maternal switching mechanism in apterous aphids. *J. Insect Physiol.* **9**, 153-164.
- LEES, A. D. (1964) The location of the photoperiodic receptors in the aphid *Megoura viciae*. *J. exp. Biol.* **41**, 119-133.
- LEES, A. D. (1965) Is there a circadian component in the *Megoura* photoperiodic clock? In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 351-356. North-Holland, Amsterdam.
- LEES, A. D. (1966a) Photoperiodic timing mechanisms in insects. *Nature, Lond.* **210**, 986-989.
- LEES, A. D. (1966b) The control of polymorphism in aphids. *Adv. Insect Physiol.* **3**, 207-277.
- LEES, A. D. (1967a) The diversity of biological clocks in aphids. In *Insects and Physiology* (Ed. BEAMENT, J. W. L. and TREHERNE, J. E.), pp. 89-99. Oliver & Boyd, Edinburgh and London.
- LEES, A. D. (1967b) Direct and indirect effects of daylength on the aphid *Megoura viciae* Buckton. *J. Insect Physiol.* **13**, 1781-1785.
- LEES, A. D. (1968) Photoperiodism in insects. In *Photophysiology*, vol. IV (Ed. GIESE, A. C.), pp. 47-137. Academic Press, New York.
- LEES, A. D. (1970) *Insect clocks and timers*. Inaugural Lecture, Imperial College of Science and Technology, 1st December 1970.
- LEES, A. D. (1971a) The relevance of action spectra in the study of insect photoperiodism. In *Biochronometry* (Ed. MENAKER, M.), pp. 372-380. National Academy of Sciences, Washington.
- LEES, A. D. (1971b) The role of circadian rhythmicity in photoperiodic induction in animals. *Proc. Int. Symp. Circadian Rhythmicity* (Wageningen, 1971), pp. 87-110.
- LEES, A. D. (1973) Photoperiodic time measurement in the aphid *Megoura viciae*. *J. Insect Physiol.* **19**, 2279-2316.
- LEES, A. D. (1977) Action of juvenile hormone mimics on the regulation of larval-adult and alary polymorphism in aphids. *Nature, Lond.* **267**, 46-48.
- LEES, A. D. (1981) Action spectra for the photoperiodic control of polymorphism in the aphid *Megoura viciae*. *J. Insect Physiol.* **27**, 761-771.
- LEES, A. D. (1983) The endocrine control of polymorphism in aphids. In: *Endocrinology of Insects*, pp. 369-377.
- LEES, A. D. (1984) temperature and the hourglass photoperiodic clock in the vetch aphid. *J. Physiol.* **351**, 50.
- LEES, A. D. (1986) Some effects of temperature on the hourglass photoperiod timer in the aphid *Megoura viciae*. *J. Insect Physiol.* **32**, 79-89.
- LEES, A. D. (1989) The photoperiodic response and phenology of an English strain of the pea aphid *Acyrtosiphon pisum*. *Ecol. Entomol.* **14**, 69-78.
- LEES, A. D. (1990) Dual photoperiodic timers controlling sex and female morph determination in the pea aphid *Acyrtosiphon pisum*. *J. Insect Physiol.* **36**, 585-591.
- LEHMANN, U. (1977) Stochastic principles in the temporal control of activity behaviour. *Int. J. Chronobiol.* **4**, 223-226.
- LEITCH, B., LAURENT, G. and SHEPHERD, D. (1992) Embryonic development of synapses on spiking local interneurons in locust. *J. Comp. Neurology* **324**, 213-236.
- LELOUP J.C., and GOLDBETER, A. (2000) Modeling the molecular regulatory mechanism of circadian rhythms in *Drosophila*. *BioEssays* **22**, 84-93.
- LELOUP, J.-C., GONZE, D. and GOLDBETER, A. (1999) Limit cycle models for circadian rhythms based on transcriptional regulation in *Drosophila* and *Neurospora*. *J. Biol. Rhythms* **14**, 433-448.
- LEPPLA, N.C., KOEHLER, P.G. and AGEE, H.R. (1989) Circadian rhythms of the German cockroach (Dictyoptera: Blattellidae): locomotion in response to different photoperiods and wavelengths of light. *J. Insect Physiol.* **35**, 63-66.
- LEUTHOLD, R. (1966) Die Bewegungsaktivität der weiblichen Schabe *Leucophaea maderae* (F.) im Laufe des Fortpflanzungszyklus und ihre experimentelle Beeinflussung. *J. Insect Physiol.* **12**, 1303-1331.
- LEVINE, R.B. and TRUMAN, J.W. (1983) Peptide activation of a simple neural circuit. *Brain Research* **279**, 335-338.
- LEWIS, C. B. and BLETCHELY, J. D. (1943) The emergence rhythm of the dung-fly *Scatophaga stercoraria* (L.). *J. anim. Ecol.* **12**, 11-18.
- LEWIS, R. D. (1976) The circadian rhythm of the weta *Hemideina thoracica* (Orthoptera): free-running rhythms, circadian rule and light entrainment. *Int. J. Chronobiol.* **3**, 241-254.
- LEWIS, R.D. (1988) A feedback model for an insect circadian clock. In: *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, Vol. 10 Part 4, (Eds. HARRIS, G. and WALKER, S.) pp. 1816-1817. The Institute of Electrical and Electronic Engineers, New York.

- LEWIS, R.D. (1990) Feedback models for biological clocks of insects. In *Trends in Biological Cybernetics*. (Ed. MENON, J.), pp. 242-269. ResearchTrends, T.C. Sreekanteswaram, Trivandrum.
- LEWIS, R.D. (1994) Modelling the circadian system of the weta, *Hemideina thoracica* (Orthoptera: Stenopelmaticidae). *J. Roy. Soc. New Zealand* **24**, 395-421.
- LEWIS, R.D., BULLIVANT, A.G. and KING, V.M. (1991) A dual pacemaker model for the circadian system of the insect *Hemideina thoracica*. *J. interdisc. Cycle Res.* **22**, 293-309.
- LEWIS, R.D. and SAUNDERS, D.S. (1987) A damped circadian oscillator model of an insect photoperiodic clock. I. Description of the model based on a feedback control system. *J. theoret. Biol.* **128**, 47-59.
- LEWIS, R.D. and WADDELL, B. (1987) A study of the location of the circadian clock of the insect *Hemideina thoracica* (Orthoptera: Stenopelmaticidae). In *Chronobiology and Chronomedicine Basic Research and Applications*, (Eds. HILDERBRANDT, G., MOOG, R., and RASCHKE, F.), Peter Lang, Frankfurt am Main-Bern-New York-Paris pp. 65-68.
- LEWIS, R.D., WARMAN, G.R. and SAUNDERS, D.S. (1997) Simulations of free-running rhythms, light entrainment and the light-pulse phase response curves for the locomotor activity rhythm in period mutants of *Drosophila melanogaster*. *J. theoret. Biol.* **185**, 503-510.
- LEWIS, T. and TAYLOR, L. R. (1965) Diurnal periodicity of flight by insects. *Trans. R. ent. Soc. Lond.* **116**, 393-479.
- LEZZI, M., GATZKA, F., INEICHEN, H. and GRUZDEV, A.D. (1991) Transcriptional activation of puff site 1-18C of *Chironomus tentans*. Hormonal responsiveness changes in parallel with diurnal decondensation cycle. *Chromosoma* **100**, 235-241.
- L'HELIAS, C. (1962) Corrélations entre les ptérines et le photoperiodisme dans la regulation du cycle sexuel chez les pucerons. *Bull. Biol. France et Belge*, **96**, 187-198.
- L'HELIAS, C., CALLEBERT, J., BEAUDRY, P. and LAUNAY, J.M. (1995) N-acetyl transferase activity during the photoperiodic-dependent *Pieris brassicae* development. *J. Insect Physiol.* **41**, 827-835.
- LHERMETTE, C.M. (1977) Investigation of the endocuticular growth layers of the wasp *Paravespula vulgaris* and application of this ageing technique to the population of a nest of *P. vulgaris*. Bachelor of Science Thesis, Bristol University.
- LIANG, D. and SCHAL, C. (1990) Circadian rhythmicity and development of the behavioural response to sex pheromone in male brown-banded cockroaches, *Supella longipalpa*. *Physiol. Entomol.* **15**, 155-361.
- LIBBY, J.L. (1961) The nervous system of certain abdominal segments and the innervation of the male reproductive system and genitalia of *Hyalophora cecropia* (Lepidoptera: Saturniidae). *Ann. Ent. Soc. Am.* **54**, 887-896.
- LIN, T.M. and LEE, H.J. (1996) The expression of locomotor circadian rhythm in female German cockroach, *Blattella germanica* (L.). *Chronobiol. Intern.* **13**, 81-91.
- LIN, T.M. and LEE, H.J. (1998) Parallel control mechanisms underlying locomotor activity and sexual receptivity of the female German cockroach, *Blattella germanica* (L.). *J. Insect Physiol.* **44**, 1039-1051.
- LINDAUER, M. (1957) Sonnenorientierung der Bienen unter der Äquatorsonne und zur Nachtzeit *Naturwissenschaften*, **44**, 1-6.
- LINDAUER, M. (1959) Angeborene und erlernte Komponenten in der Sonnenorientierung der Bienen. *Z. vergl. Physiol.* **42**, 43-62.
- LINDAUER, M. (1960) Time-compensated sun orientation in bees. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 371-377.
- LINN, C.E. (1997) Neuroendocrine factors in the photoperiodic control of male moth responsiveness to sex sex pheromone. In: Carde, R.T. and Minks, A.K. (Eds.). *Insect Pheromone Research. New Directions*. Chapman and Hall, New York, pp. 194-209.
- LINN, C.E., and ROELOFS, W.L. (1986) Modulatory effects of octopamine and serotonin on male sensitivity and and periodicity to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* **3**, 161-171.
- LINN, C.E. and ROELOFS, W.L. (1992) Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. *Arch. Insect Biochem. Physiol.* **20**, 285-302.
- LINN, C.E., CAMPBELL, M.G. and ROELOFS, W.L. (1992) Photoperiodic cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male cypsy moths, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* **20**, 265-284.
- LINN, C.E., CAMPBELL, M.G., POOLE, K.R., WU, W.-Q. and ROELOFS, W.L. (1996) Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: relationship to the modulatory action of octopamine. *J. Insect Physiol.* **42**, 881-891.
- LINN, C.E., POOLE, K.R., WU, W.-Q., and ROELOFS, W.L. (1995) Circadian changes in melatonin in the

- nervous system and haemolymph of the cabbage looper moth, *Trichoplusia ni*. *J. Comp. Physiol. A* **176**, 761-771.
- LINS, F. and ELSNER, N. (1995) Descending stridulatory interneurons in the suboesophageal ganglion of two grasshopper species: II. Influence upon the stridulatory patterns. *J. Comp. Physiol. A* **176**, 823-833.
- LIPKOW, E. (1966) Biologisch-ökologische Untersuchungen Über *Tachyporus*-Arten und *Tachinus rufipes* (Col., Staphyl.). *Pedobiologia*, **6**, 140-177.
- LIPTON, G. R. and SUTHERLAND, D. J. (1970) Activity rhythms in the American cockroach, *Periplaneta americana*. *J. Insect Physiol.* **16**, 1555-1566.
- LIU, X., LORENZ, L., YU, Q., HALL, J.C. and ROSBASH, M. (1988) Spatial and temporal expression of the period gene in *Drosophila melanogaster*. *Genes Devel.* **2**, 228-238.
- LLOYD, D. and LLOYD, L. (1994) Hypothesis: a controlled chaotic attractor constitutes the central oscillator of the circadian clock. Bulletin du Groupe d'Etude des Rythmes biologiques, Tome 26, no 7 pp.188-192.
- LOEB, M. (1982) Diapause and development in the tobacco budworm, *Heliothis virescens*: a comparison of haemolymph ecdysteroid titres. *J. Insect Physiol.* **28**, 667-673.
- LOEB, M.J., BRANDT, E.P. and BIRNBAUM, M.J. (1984) Ecdysteroid production by testes of the tobacco budworm, *Heliothis virescens*, from last larval instar to adult. *J. Insect Physiol.* **30**, 375-381.
- LOEB, M.J., BRANDT, E.P., WOODS, C.W. and BELL, R.A. (1988) Secretion of ecdysteroid by sheaths of testes of the gypsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. *J. Exp. Zool.* **248**, 94-100.
- LOHER, W. (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* Walker. *J. comp. Physiol.* **79**, 173-190.
- LOHER, W. (1979) Circadian rhythmicity of locomotor behavior and oviposition in female *Teleogryllus commodus*. *Behav. Ecol. Sociobiol.* **5**, 253-262.
- LOHER, W. (1980) The effect of male calling on female locomotor activity of *Teleogryllus commodus*. *Behav. Ecol. Sociobiol.* **5**, 383-390.
- LOHER, W. and CHANDRASHEKARAN, M. K. (1970) Circadian rhythmicity in the oviposition of the grasshopper *Chorthippus curtipennis*. *J. Insect Physiol.* **16**, 1677-1688.
- LOHER, W. and ZERVAS, G. (1979) The mating rhythm of the olive fruit fly, *Dacus oleae*. *Zeits. fuer Ang. Ent.* **88**, 425-435.
- LOHMANN, M. (1964) Der einfluss von Beleuchtungsstärke und Temperatur auf die Tagesperiodische Laufaktivität des Mehlkäfers, *Tenebrio molitor* L. *Z. vergl. Physiol.* **49**, 341-389.
- LOHMANN, M. (1967) Ranges of circadian period length. *Experientia*, **23**, 788-790.
- DE LOOF, A., VAN LOON, J. and VANDERROOST, C. (1979) Influence of ecdysterone, precocene and compounds with juvenile hormone activity on induction, termination and maintenance of diapause in the parasitoid wasp, *Nasonia vitripennis*. *Physiol. Ent.* **4**, 319-328.
- LOUNIBOS, L. P. and BRADSHAW, W. E. (1975) A second diapause in *Wyeomyia smithii*: seasonal incidence and maintenance by photoperiod. *Can. J. Zool.* **53**, 215-221.
- LUKAT, R. (1978) Circadian growth layers in cuticle of behaviourally arrhythmic cockroaches (*Blaberus fuscus*, Insecta, Blattodea). *Experientia*, **34**, 477.
- LUKAT, R., WEBER, F. and WIEDENMANN, G. (1989) Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a decentral circadian "clock"? *J. Insect Physiol.* **35**, 321-329.
- LUM, P. T. M., NAYAR, J. K. and PROVOST, M. W. (1968) The pupation rhythm in *Aedes taeniorhynchus* III. Factors in developmental synchrony. *Ann. ent. Soc. Am.* **61**, 889-899.
- LUMME, J. (1978) Phenology and photoperiodic diapause in northern populations of *Drosophila*. In *Evolution of Insect Migration and Diapause* (Ed. DINGLE, H.), pp. 145-170. Springer-Verlag, New York, Heidelberg, Berlin.
- LUMME, J. (1981) Localization of the genetic unit controlling the photoperiodic adult diapause in *Drosophila littoralis*. *Hereditas* **94**, 241-244.
- LUMME, J. and KERÄNEN, L. (1978) Photoperiodic diapause in *Drosophila lummei* Hackman is controlled by an X-chromosomal factor. *Hereditas* **89**, 261-262.
- LUMME, J., LAKOVAARA, S. and SAURA, A. (1972) The influence of daylength and temperature on the testis pterin content of *Drosophila littoralis*. *J. Insect Physiol.* **18**, 2043-2053.
- LUMME, J. and OIKARINEN, A. (1977) The genetic basis of geographically variable photoperiodic diapause in *Drosophila littoralis*. *Hereditas* **86**, 129-142.
- LUMME, J. and POHJOLA, L. (1980) Selection against photoperiodic diapause started from monohybrid crosses in *Drosophila littoralis*. *Hereditas* **92**, 377-378.
- LUTZ, F. E. (1932) Experiments with Orthoptera concerning diurnal rhythms. *Ann. Mus. Novitates*, **550**, 1-24.

- LUTZ, P. E. and JENNER, C. E. (1964) Life-history and photoperiodic response of nymphs of *Tetragoneuria cynosura* (Say). *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **127**, 304-316.
- MA, P.W.K., GARDEN, R.W., NIERMANN, J.T., O'CONNOR, M., SWEEDLER, J.V. and ROELOFS, W.L. (2000) Characterizing the Hez-PBAN gene products in neuronal clusters with immunocytochemistry and MALDI MS. *J. Insect Physiol.* **46**, 221-230.
- MA, P.W.K., KNIPPLE, D.C. and ROELOFS, W.L. (1998) Expression of a gene that encodes pheromone biosynthesis activating neuropeptide in the central nervous system of corn earworm, *Helicoverpa zea*. *Insect Biochem. Mol. Biol.* **28**, 373-385.
- MA, P.W.K. and ROELOFS, W.L. (1995) Sites of synthesis and release of PBAN-like factor in the female European corn borer, *Ostrinia nubilalis*. *J. Insect Physiol.* **41**, 339-350.
- MacLEOD, E. G. (1967) Experimental induction and elimination of adult diapause and autumnal coloration in *Chrysopa carnea* (Neuroptera). *J. Insect Physiol.* **13**, 1343-1349.
- MACK, J. and ENGELMANN, W. (1981) Circadian control of the locomotor activity in eye mutants of *Drosophila melanogaster*. *J. interdisc. Cycle Res.* **12**, 313-323.
- MAIER, R. W. (1973) Phase-shifting of the circadian rhythm of eclosion in *Drosophila pseudoobscura* with temperature pulses. *J. interdiscipl. Cycle Res.* **4**, 125-135.
- MANSINGH, A. (1971) Physiological classification of dormancies in insects. *Can. Ent.* **103**, 983-1009.
- MANSINGH, A. and SMALLMAN, B. N. (1966) Photoperiod control of an 'obligatory' pupal diapause. *Can. Ent.* **98**, 613-616.
- MANSINGH, A. and SMALLMAN, B. N. (1971) The influence of temperature on the photoperiodic regulation of diapause in Saturniids. *J. Insect Physiol.* **17**, 1735-1739.
- MARCOVITCH, S. (1923) Plant lice and light exposure. *Science, Wash.* **58**, 537-538.
- MARCOVITCH, S. (1924) The migration of the Aphididae and the appearance of sexual forms as affected by the relative length of daily light exposure. *J. Agric. Res.* **27**, 513-522.
- MARTIN, H. and MARTIN, U. (1987) Transfer of a time-signal isochronon with local time in translocation experiments to the geographical longitude. *J. comp. Physiol. A* **160**, 3-9.
- MARTIN, U., MARTIN, H. and LINDAUER, M. (1978) Transplantation of a time-signal in honey bees. *J. comp. Physiol.* **124**, 193-201.
- MARTINEZ, T. and CAMPS, F. (1988) Stimulation of sex pheromone production by head extract in *Spodoptera littoralis* at different times of the photoperiod. *Arch. Insect Biochem. Physiol.* **9**, 211-220.
- MASAKI, S. (1956) The local variation in the diapause pattern of the cabbage moth, *Barathra brassicae* Linné, with particular reference to the aestival diapause (Lepidoptera: Noctuidae). *Bull. Fac. Agr. Mie Univ.* **13**, 29-46.
- MASAKI, S. (1957) Ecological significance of diapause in the seasonal cycle of *Abraxas miranda*. *Btl. Bull. Fac. Agr. Mie Univ.* **15**, 15-24.
- MASAKI, S. (1958) The response of a 'short-day' insect to certain external factors: the induction of diapause in *Abraxas miranda* Butl. *Japan. J. appl. Ent. Zool.* **2**, 285-294.
- MASAKI, S. (1959) Seasonal changes in the mode of diapause in the pupae of *Abraxas miranda* Butler (Lepidoptera: Geometridae). *Bull. fac. Agric. Hirosaki Univ.* **5**, 14-27.
- MASAKI, S. (1968) Geographic adaptation in the seasonal life cycle of *Mamestra brassicae* (Linné) (Lepidoptera: Noctuidae). *Bull. Fac. Agr. Hirosaki Univ.* **14**, 16-26.
- MASAKI, S. (1973) Climatic adaptation and photoperiodic response in the band-legged ground cricket. *Evolution*, **36**, 587-600.
- MASAKI, S. (1978) Seasonal and latitudinal adaptations in life cycles of crickets. In: *Evolution of Insect Migration and Diapause*. Ed: H. Dingle. Springer-Verlag. Pp. 72-100.
- MASAKI, S. (1980) Summer diapause. *Ann. Rev. Ent.* **25**, 1-25.
- MASAKI, S. (1996) Geographical variation of life cycle in crickets (Ensifera: Grylloidea). *Eur. J. Entomol.* **93**, 281-302.
- MASAKI, S., IGARASHI, R., WATARI, Y. and FUJIBAYASHI, Y. (1992) Photoperiodic time measurement in seasonal adaptations of ground crickets. In *Circadian Clocks from Cell to Human*, ed: T. Hiroshige and K. Honma. Hokkaido Univ. Press, Sapporo. Pp. 73-88.
- MASAKI, S. and KIKAKAWA, S. (1981) The diapause clock in a moth: response to temperature signals. In *Biological Clocks in Seasonal Reproductive Cycles*. Ed B.K. Follett and D.E. Follett, John Wright & Sons Ltd., Bristol. Pp. 101-112.
- MASAKI, S. and OYAMA, N. (1963) Photoperiodic control of growth and wing form in *Nemobius yezoensis* Shiraki (Orthoptera: Gryllidae). *Kontyu*, **31**, 16-26.

- MASAKI, S. and SAKAI, T. (1965) Summer diapause in the seasonal cycle of *Mamestra brassicae* Linné. *Japan. J. appl. Ent. Zool.* **9**, 191-205.
- MASAKI, S., UMEYA, K., SEKIGUCHI, Y. and KAWASAKI, R. (1968) Biology of *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) in Japan. III. Photoperiodic induction of diapause in relation to the seasonal life cycle. *Appl. Ent. Zool.* **3**, 55-66.
- MASLENNIKOVA, V. A. (1958) Conditions determining diapause in the parasitic Hymenoptera *Apanteles glomeratus* L. (Braconidae), and *Pteromalus puparum* (Chalcididae). *Ent. Obozr.* **37**, 538-545. (in Russian.)
- MASLENNIKOVA, V.A. (1968) The regulation of seasonal development in parasitic insects. In *Photoperiodic Adaptations in Insects and Acari* (Ed. DANILEVSKII, A. S.), pp. 129-152. Leningrad University Press. (In Russian.)
- MASLENNIKOVA, V.A. (1977) Different sensitivity of tissues of diapausing and non-diapause larvae of *Calliphora vicina* R.-D. to β -ecdysone. *Akad. Nauk. USSR Dokl.* **234**, 1213-1216.
- MASLER, E.P., RAINA, A.K., WAGNER, R.M. and KOCHANSKY, J.P. (1994) Isolation and identification of a pheromonotropic neuropeptide from the brain-suboesophageal ganglion complex of *Lymantria dispar*: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* **24**, 829-836.
- MATSUMOTO, S., KITAMURA, A., NAGASAWA, H., KATAOKA, H., ORIKASE, C., MITSUI, T. and SUZUKI, A. (1990) Functional diversity of a neurohormone produced by the suboesophageal ganglion; molecular identity of melanization and reddish colorization hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol.* **36**, 427-432.
- MCCABE, C. and BIRLEY, A. (1998) Oviposition in the period genotypes of *Drosophila melanogaster*. *Chronobiol. Intern.* **15**, 119-133.
- MCCLELLAND, G. A. H. and GREEN, C. A. (1970) Subtle periodicity of pupation in rapidly developing mosquitoes. *Bull. Wld Hlth Org.* **42**, 951-955.
- MCCLEOD, D.G.R. and BECK, S.D. (1963) Photoperiodic termination of diapause in an insect. *Biol. Bull. Mar. Biol. Lab., Woods Hole* **124**, 84-96.
- MCDONALD M.J. and ROSBASH, M. (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107**, 567-578.
- MCELROY, W.D. and DeLUCA, M. (1985) Biochemistry of insect luminescence. In: Kerkut, G.A. and Gilbert, L.I. (Eds.) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Pergamon Press, Oxford, pp. 553-563.
- McHAFFEY, D. G. and HARWOOD, R. F. (1970) Photoperiod and temperature influences on diapause in eggs of the floodwater mosquito *Aedes dorsalis* Meigen (Diptera, Culicidae). *J. Med. Ent.* **7**, 631-644.
- McNABB, S.L., BAKER, J.D., AGAPITE, J., STELLER, H., RIDDIFORD, L.M. and TRUMAN, J.W. (1997) Disruption of behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. *Neuron* **19**, 813-823.
- McNAMARA, P., SEO, S.B., RUDIC, R.D., SEGHALI, A., CHAKRAVARTI, D. and FITZGERALD, G.A. (2001) Regulation of CLOCK and MOP4 by nuclear hormone receptors in vasculature: A humoral mechanism to reset a peripheral clock. *Cell* **105**, 877-889.
- McNEIL, J.N. and FIELDS, P.G. (1985) Seasonal diapause development and diapause termination in the European skipper, *Thymelicus lineola* (Ochs.). *J. Insect Physiol.* **31**, 467-470.
- McPHERSON, J. E. (1974) Photoperiod effects in a southern Illinois population of the *Euscistus tristigmus* complex (Hemiptera: Pentatomidae). *Ann. ent. Soc. Am.* **67**, 943-952.
- McPHERSON, J. E. (1978) Sensitivity of immature *Thyanta calceata* (Hemipter: Pentatomidae) to photoperiod as reflected by adult color and pubescence. *Gt. Lakes Ent.* **11**, 71-76.
- McWATTERS, H.G. and SAUNDERS, D.S. (1996) The influence of each parent and geographic origin on larval diapause in the blow fly, *Calliphora vicina*. *J. Insect Physiol.* **42**, 721-726.
- McWATTERS, H.G. and SAUNDERS, D.S. (1997) Inheritance of the photoperiodic response controlling larval diapause in the blow fly, *Calliphora vicina*. *J. Insect Physiol.* **43**, 709-717.
- MBATA, G.N. and RAMASWAMY, S.B. (1990) Rhythmicity of sex pheromone content in female *Heliothis virescens*: Impact of mating. *Physiol. Entomol.* **15**, 423-432.
- MEDER, E. (1958) Über die Eiberechnung der Sonnenwanderung bei der Orientierung der Honigbiene. *Z. vergl. Physiol.* **40**, 610-64.
- MEDUGORAC, I. (1967) Orientierung der Bienen in Raum und Zeit nach Dauernarkose. *Z. Bienenforsch.* **9**, 105-119.
- MEDUGORAC, I. and LINDAUER, M. (1967) Das Zeitgedächtnis der Bienen unter dem Einfluss von Narkose und von sozialen Zeitgebern. *Z. vergl. Physiol.* **55**, 450-474.

- MEIJER, J.H., DAAN, S., OVERKAMP, G.F.J. and HERMANN, P.M. (1990) The two-oscillator circadian system of tree shrews (*Tupaia belangeri*) and its response to light and dark pulses. *J. Biol. Rhythms* **5**, 1-16.
- MEINHERTZHAGEN, I.A. and PYZA, E. (1996) Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. *Trends in Neuroscience* **19**, 285-291.
- MEINHERTZHAGEN, I.A. and PYZA, E. (1999) Neurotransmitter regulation of circadian structural changes in the fly's visual system. *Microscop. Res. Tech.* **45**, 96-105.
- MELCHERS, G. (1956) Die Beteiligung der endonomen Tagesrhythmik am Zustandekommen der photoperiodischen Reaktionen der Kurztagpflanze *Kalanchoe blossfeldiana*. *Z. Naturf.* **11b**, 544-548.
- MELLANBY, K. (1940) The daily rhythm in the cockroach *Blatta orientalis* II. Observations and experiments on a natural infestation. *J. exp. Biol.* **17**, 278-285.
- MENAKER, M. (1978) The physiology of circadian pacemakers. *Ann. Rev. Physiol.* **40**, 501-526.
- MENAKER, M. and GROSS, G. (1965) Effects of fluctuating temperature on diapause induction in the pink bollworm. *J. Insect Physiol.* **11**, 911-914.
- MENAKER, M. and ZIMMERMAN, N. (1976) Role of the pineal in the circadian system of birds. *Amer. Zool.* **16**, 45-55.
- MESSINGER, P. S. (1964) The influence of rhythmically fluctuating temperatures on the development and reproduction of the spotted alfalfa aphid, *Therioaphis maculata*. *J. econ. Ent.* **57**, 71-76.
- MEUDEC, M. (1966) Influence de la température et de la lumière pendant le développement sur l'état ovarien à l'éclosion chez *Acrolepia assectella* Zeller (Insecte lépidoptère). *C. R. Acad. Sci.* **263**, 554-557.
- MEYER-ROCHOW, V.B. and BROWN, P.J. (1998) Possible natural circaseptan rhythm in the beach beetle *Chaerodes trachyscelides* White. *Acta Neurobiol. Exp.* **58**, 287-290.
- MILLER, T.A. (1975) Insect visceral muscle. In: Usherwood, P.N.R. (Ed.). *Insect Muscle*. Academic Press, New York, pp. 545-606.
- MINIS, D. H. (1965) Parallel peculiarities in the entrainment of a circadian rhythm and photoperiodic induction in the pink bollworm (*Pectinophora gossypiella*). In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 333-343. North-Holland, Amsterdam.
- MINIS, D. H. and PITTENDRIGH, C. S. (1968) Circadian oscillation controlling hatching: its ontogeny during embryogenesis of a moth. *Science, Wash.* **159**, 534-536.
- MIZOGUCHI, A. and ISHIZAKI, H. (1982) Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. Nat. Acad. Sci. USA* **79**, 2726-2730.
- MIZOGUCHI, A. and ISHIZAKI, H. (1984a) Circadian clock controlling gut-purge rhythm of the saturniid *Samia cynthia ricini*: its characterization and entrainment mechanism. *J. Comp. Physiol. A* **155**, 639-647.
- MIZOGUCHI, A. and ISHIZAKI, H. (1984b) Further evidence for the presence of a circadian clock in the prothoracic glands of the saturniid moth *Samia cynthia ricini*: decapitated larvae can respond to light-dark changes. *Develop. Growth and Differentiation* **26**, 607-611.
- MIZOGUCHI, A., OHASHI, Y., HOSODA, K., ISHIBASHI, J. and KATAOKA, H. (2001) Developmental profile of the changes in the prothoracicotropic hormone titre in haemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol.* **31**, 349-358.
- MOORE, D. and RANKIN, M.A. (1985) Circadian locomotor rhythms in individual honeybees. *Physiol. Entomol.* **10**, 191-197.
- MOORE, D., SIEGFRIED, D., WILSON, R. and RANKIN, M.A. (1989) The influence of time of day on the foraging behavior of the honey bee, *Apis mellifera*. *J. Biol. Rhythms* **4**, 305-325.
- MORIARTY, F. (1959) The 24-hr rhythm of emergence of *Ephesia kühniella* Zell. from the pupa. *J. Insect Physiol.* **3**, 357-366.
- MORITA, A. and NUMATA, H. (1997) Distribution of photoperiodic receptors in the compound eye of the bean bug, *Riptortus clavatus*. *J. comp. Physiol. A* **180**, 181-185.
- MORITA, A. and NUMATA, H. (1999) Localization of the photoreceptor for photoperiodism in the stink bug, *Plautia stali*. *Physiol. Entomol.* **24**, 189-195.
- MORRIS, M.C. and TAKEDA, S. (1994) The adult eclosion rhythm in *Hyphantria cunea* (Lepidoptera: Arctiidae): endogenous and exogenous light effects. *Biological Rhythm Res.* **25**, 464-476.
- MORTON, D.B. and TRUMAN, J.W. (1988) The EGPs- the eclosion hormone and cyclic GMP regulated phosphoproteins. II. Regulation of appearance by the steroid hormone 20-hydroxyecdysone in *Manduca sexta*. *J. Neuroscience* **8**, 1338-1345.
- MOSHITZKY, P., FLEISCHMANN, I., CHAIMOV, N., SAUDAN, P., KLAUSER, S., KUBLI, E. and APPLEBAUM, S.W. (1996) Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch. Insect Biochem. Physiol.* **32**, 363-374.
- MOTE, M. I. and BLACK, K.R. (1981) Action spectrum and threshold sensitivity of entrainment of circadian running activity in the cockroach *Periplaneta americana*. *Photochem. Photobiol.* **34**, 257-265.

- MROSOVSKY, N., REEBS, S.G., HONRADO, G.I. and SALMON, P.A. (1989) Behavioral entrainment of circadian rhythms. *Experientia* **45**, 696-702.
- MROSOVSKY, N. and SALMON, P.A. (1987) A behavioural method for accelerating re-entrainment of rhythms to new light-dark cycles. *Nature* **330**, 372-373.
- MÜLLER, H. J. (1954) Der Saisondimorphismus bei Zikaden der Gattung *Euscelis* Brullé. *Beitr. Ent.* **4**, 1-56.
- MÜLLER, H. J. (1955) Die Saisonformenbildung von *Araschnia levana*, ein photoperiodisch gesteuerter Diapause-effekt. *Naturwissenschaften*, **42**, 134-135.
- MÜLLER, H. J. (1957) Die Wirkung exogener Faktoren auf die Zyklische Formenbildung der Insekten, insbesondere der Gattung *Euscelis* (Hom., Auchenorrhyncha). *Zool. Jb., Syst.* **85**, 317-430.
- MÜLLER, H. J. (1960) Die Bedeutung der Photoperiode im Lebensablauf der Insekten. *Z. angew. Ent.* **47**, 7-24.
- MÜLLER, H. J. (1962a) Über den Saisondimorphen entwicklungszyklus und die Aufhebung der Diapause bei *Aleurochiton companatus* (Baerensprung) (Homoptera, Aleyrodidae). *Entomologia exp. appl.* **5**, 124-138.
- MÜLLER, H.J. (1962b) Über die Induktion der Diapause und der Ausbildung der Saisonformen bei *Aleurochiton complanatus* (Baerensprung). *Z.Morph.Ökol.Tiere*, **51**, 575-610.
- MÜLLER, H. J. (1964) Über die Wirkung Verschiedener Spektralbereiche bei der photoperiodischen Induktion der Saisonformen von *Euscelis plebejus* Fall. (Homoptera: Jassidae). *Zool. Jb. Abt. Allgem. Zool. Physiol. Tiere*, **70**, 411-426.
- MÜLLER, H. J. (1970) Formen der Dormanz bei Insekten. *Nova Acta Leopold*, **35**, 7-27.
- MÜLLER, K. (1973) Circadian rhythms of locomotor activity in aquatic organisms in the sub-arctic summer. *Aquilo, Ser. Zool.* **14**, 1-18.
- MULLINS, D.E., KEIL, C.B. and WHITE, R.H. (1992) Maternal and paternal nitrogen investment in *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *J. Exp. Biol.* **162**, 55-72.
- MUNRO, J. (1972) Terminal diapause in geometrid pupae – a new link in the endocrine chain? In: *Insect Endocrines*, Eds: Novak, V.J.A. and Slama, K. Vol. 3, Academia, Prague, pp. 89-97.
- MURRAY, S.B. and NEVILLE, A.C. (1997) The role of the electrostatic coat in the formation of cholesteric liquid spherulites from alpha-chitin. *Int. J. Biol. Macromolecules* **20**, 123-130.
- MURRAY, S.B. and NEVILLE, A.C. (1998) The role of pH, temperature and nucleation in the formation of cholesteric liquid crystal spherulites from chitin and chitosan. *Int. J. Biol. Macromolecules* **22**, 137-144.
- MYBURGH, A. C. (1963) Diurnal rhythms in emergence of mature larvae from fruit and eclosion of adult *Pterandrus rosae* (Ksh.) and *Ceratitis capitata* (Wied.). *S. Afr. J. Agric. Sci.* **6**, 41-46.
- MYERS, K. (1952) Rhythms in emergence and other aspects of behaviour of the Queensland fruit-fly (*Dacus (Strumeta) tryoni* Frogg.) and the solanum fruit-fly (*Dacus (Strumeta) cacuminatus* Hering). *Aust. J. Sci.* **15**, 101-102.
- MYERS, M.P., WAGER-SMITH, K., ROTHENFLUH-HILFIKER, A., and YOUNG, M.W. (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* **271**, 1736-1740.
- NAKAMURA, K. and HODKOVA, M. (1998) Photoreception in entrainment of rhythms and photoperiodic regulation of diapause in a hemipteran, *Graphosoma lineatum*. *J. Biol. Rhythms* **13**, 159-166.
- NANDA, K.K. and HAMNER, K.C. (1958) Studies on the nature of the endogenous rhythm affecting photoperiodic response of Biloxi soy bean. *Bot. Gaz.* **120**, 14-25.
- NASH, T. A. M. and TREWERN, M. A. (1972) Hourly distribution of larviposition by *Glossina austeni* Newst. and *G. morsitans morsitans* Westw. (Dipt. Glossinidae). *Bull. ent. Res.* **61**, 693-700.
- NASSEL, D.R. (2000) Functional roles of neuropeptides in the insect central nervous system. *Naturwiss.* **87**, 439-449.
- NÄSSEL, D.R., SHIGA, S., MOHRHERR, C.J. and RAO, K.R. (1993) Pigment dispersing hormone-like peptide in the nervous system of the flies *Phormia* and *Drosophila*: immunocytochemistry and partial characterization. *J. comp. Neurol.* **331**, 183-198.
- NÄSSEL, D.R., SHIGA, S., WIKSTRAND, E.M. and RAO, K.R. (1991) Pigment dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual systems. *Cell Tissue Res.* **266**, 511-523.
- NAYAR, J. K. (1967a) Endogenous diurnal rhythm of pupation in a mosquito population. *Nature, Lond.* **214**, 828-829.
- NAYAR, J. K. (1967b) The pupation rhythm in *Aedes taeniorhynchus* (Diptera: Cuficidae). II. Ontogenetic timing, rate of development, and endogenous diurnal rhythm of pupation. *Ann. ent. Soc. Am.* **60**, 946-971.
- NAYAR, J. K. (1968) The pupation rhythm in *Aedes taeniorhynchus* IV. Further studies of the endogenous diurnal (circadian) rhythm of pupation. *Ann. ent. Soc. Am.* **61**, 1408-1417.

- NAYAR, J. K. (1972) Effects of fluctuating temperatures on life span of *Aedes taeniorhynchus* adults. *J. Insect Physiol.* **18**, 1303-1313.
- NAYAR, J. K. and SAUERMAN, D. M. (1971) The effect of light regimes on the circadian rhythm of flight activity in the mosquito *Aedes taeniorhynchus*. *J. exp. Biol.* **54**, 745-756.
- NECHOLS, J.R., TAUBER, M.J. and TAUBER, C.A. (1987) geographical variability in ecophysiological traits controlling dormancy in *Chrysopa oculata* (Neuroptera: Chrysopidae). *J. Insect Physiol.* **33**, 627-633.
- NEUMANN, D. (1963) Über die Steuerung der lunaren Schwärmpseudoperiodik der Mücke *Clunio marinus*. *Verh. dt. Zool. Ges. Wien* 1962, pp. 275-285.
- NEUMANN, D. (1966a) Die intraspezifische Variabilität der lunaren und täglichen Schlüpfzeiten von *Clunio marinus* (Diptera: Chironomidae). *Verh. dt. Zool. Ges. Jena* 1965, pp. 223-233.
- NEUMANN, D. (1966b) Die lunare und tägliche Schlüpfperiodik der Mücke *Clunio*. Steuerung und Abstimmung auf die Gezeitenperiodik. *Z. vergl. Physiol.* **53**, 1-61.
- NEUMANN, D. (1967) Genetic adaptation in emergence time of *Clunio* populations to different tidal conditions. *Helgoländer wiss. Meeresunters.* **15**, 163-171.
- NEUMANN, D. (1971) Eine nicht-reziproke Kruzungssterilität zwischen ökologischen Rassen der Mücke *Clunio marinus*. *Oecologia, Berl.* **8**, 1-20.
- NEUMANN, D. (1976a) Adaptations of chironomids to intertidal environments. *Ann. Res. Ent.* **21**, 387-414.
- NEUMANN, D. (1976b) Entrainment of a semilunar rhythm. In: *Biological Rhythms in the Marine Environment* (Ed. DECOURSEY, P. J.), pp. 115-127. Univ. South Carolina Press, Columbia, S.C.
- NEUMANN, D. (1988) Temperature compensation of circalunar timing in the intertidal insect *Clunio*. *J. comp. Physiol. A* **163**, 671-676.
- NEUMANN, D. (1989) Circadian components of semilunar and lunar timing mechanisms. *J. Biol. Rhythms* **4**, 285-294.
- NEUMANN, D. and HEIMBACH, F. (1984) Time cues for semilunar reproduction rhythms in European populations of *Clunio marinus*. II. The influence of tidal temperature cycles. *Biol. Bull.* **166**, 509-524.
- NEUMANN, D. and HEIMBACH, F. (1985) Circadian range of entrainment in the semilunar eclosion rhythm of the marine insect *Clunio marinus*. *J. Insect Physiol.* **31**, 549-557.
- NEUMANN, D. and HONEGGER, W. (1969) Adaptations of the intertidal midge *Clunio* to arctic conditions. *Oecologia, Berl.* **3**, 1-13.
- NEUMANN, D., KAMINSKY, R. and HEIMBACH, F. (1997) Timing of eclosion in marine insects on Mediterranean shores in studies on *Clunio mediterraneus*, *C. ponticus* and *Thalassomyia frauenfeldi* (Diptera: Chironomidae). *Marine Biol.* **129**, 513-521.
- NEUMANN, D. and KRÜGER, M. (1985) Combined effects of photoperiod and temperature on the diapause of an intertidal chironomid. *Oecologia* **67**, 154-156.
- NEUMANN, D. and SPINDLER, K.-D. (1991) Circasemilunar control of imaginal disc development in *Clunio marinus*: temporal switching point, temperature compensated developmental time and ecdysteroid profile. *J. Insect Physiol.* **37**, 101-109.
- NEVILLE, A.C. (1963) Daily growth layers in locust rubber-like cuticle, influenced by an external rhythm. *J. Insect Physiol.* **9**, 177-186.
- NEVILLE, A.C. (1965) Circadian organization of chitin in some insect skeletons. *Quart. J. Micro. Sci.* **106**, 315-325.
- NEVILLE, A.C. (1967) A dermal light sense influencing skeletal structures in locusts. *J. Insect Physiol.* **13**, 933-939.
- NEVILLE, A. C. (1970) Cuticle ultrastructure in relation to the whole insect. *Symp. R. ent. Soc. Lond.* **5**, 17-39.
- NEVILLE, A.C. (1975) *Biology of the Arthropod Cuticle*. Springer-Verlag, Berlin.
- NEVILLE, A.C. (1983) Daily cuticular growth layers and the teneral stage in adult insects: a review. *J. Insect Physiol.* **29**, 211-219.
- NEVILLE, A.C. (1986) The Physics of Helicoids: Multidirectional 'plywood' structures in biological systems. *Physical Bulletin* **37**, 74-76.
- NEVILLE, A.C. and LUKE, B.M. (1969) A two-system model for chitin-protein complexes in insect cuticles. *Tissue and Cell* **1**, 689-707.
- NEWBY, L.M. and JACKSON, F.R. (1991) *Drosophila ebony* mutants have altered circadian activity rhythms but normal eclosion. *J. Neurogenetics* **7**, 85-101.
- NEWBY, L.M. and JACKSON, F.R. (1993) A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. *Genetics* **135**, 1077-1090.
- NEWBY, L.M. and JACKSON, F.R. (1996) Regulation of a specific circadian clock output pathway by Lark, a putative RNA-binding protein with repressor activity. *J. Neurobiol.* **31**, 117-128.
- NICKELMANN, M., HOCHER, J. and TZSCHENTKE, B. (1999) Biological rhythms in birds – development

- insights and perspectives. *Comp. Biochem. Physiol. A* **124**, 429-437.
- NICKISCH-ROSENEGK, E.v., KRIEGER, J., KUBICK, S., LAAGE, R., STROBEL, J., STROTMANN, J. and BREER, H. (1996) Cloning of biogenic amine receptors from moths (*Bombyx mori* and *Heliothis virescens*). *Insect Biochem. Mol. Biol.* **26**, 817-827.
- NIELSEN, E. T. and HAEGAR, J. S. (1954) Pupation and emergence in *Aedes taeniorhynchus* (Wied.). *Bull. ent. Res.* **45**, 757-768.
- NIELSEN, J., PEIXOTO, A.A., PICCIN, A., COSTA, R., KYRIACOU, C.P. and CHALMERS, D. (1994) Big flies, small repeats: the "Thr-Gly" region of the *period* gene in Diptera. *Molec. Biol. Evol.* **11**, 839-853.
- NIJHOUT, H.F. (1981) Physiological control of moulting in insects. *Amer. Zool.* **21**, 631-640.
- NIJHOUT, H.F. (1994) *Insect Hormones*. Princeton University Press, New Jersey.
- NISHIITSUTSUJI-UWO, J., PETROPULOS, S. F. and PITTENDRIGH, C. S. (1967) Central nervous system control of circadian rhythmicity in the cockroach. I. Role of the pars intercerebralis. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **133**, 679-696.
- NISHIITSUTSUJI-UWO, J. and PITTENDRIGH, C. S. (1967) The neuroendocrine basis of mid-gut tumour induction in cockroaches. *J. Insect Physiol.* **13**, 851-859.
- NISHIITSUTSUJI-UWO, J. and PITTENDRIGH, C. S. (1968a) Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. *Z. vergl. Physiol.* **59**, 1-13.
- NISHIITSUTSUJI-UWO, J. and PITTENDRIGH, C. S. (1968b) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? *Z. vergl. Physiol.* **58**, 14-46.
- NISHIZUKA, M., AZUMA, A. and MASAKI, S. (1998) Diapause response to photoperiod and temperature in *Lepisma saccharina* Linnaeus (Thysanura: Lepismatidae). *Entomol. Sci.* **1**, 7-14.
- NITSCH, I. P. and WENT, F. W. (1959) The induction of flowering in *Xanthium pensylvanicum* under long days. In *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. WITHROW, R. B.), pp. 311-314. Am. Ass. Adv. Sci., Washington.
- NJUS, D., SULZMAN, F. M. and HASTINGS, J. W. (1974) Membrane model for the circadian clock. *Nature*, **248**, 116-120.
- NONAKA, H., EMOTO, N., IKEDA, K., FUKUYA, H., ROHMAN, M.S., RAHARJO, S.B., YAGITA, K., OKAMURA, H. and YOKOYAMA, M. (2001) Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. *Circulation* **104**, 1746-1748.
- NORONHA, K.F. and LANGE, A.B. (1997) Proctolin's role in neurally evoked contractions of the locust oviducts. *J. Neurobiol.* **33**, 139-150.
- NORRIS, K. H., HOWELL, F., HAYES, D. K., ADLER, V. E., SULLIVAN, W. N. and SCHECHTER, M. S. (1969) The action spectrum for breaking diapause in the codling moth, *Laspeyresia pomonella* (L.) and the oak silkworm, *Antheraea pernyi* Guer. *Proc. natn. Acad. Sci. U.S.A.* **63**, 1120-1127.
- NORRIS, M.J. (1959) The influence of day-length on imaginal diapause in the red locust *Nomadacris septemfasciata* (Serv.). *Entomologia exp. appl.* **2**, 154-168.
- NORRIS, M.J. (1962) Diapause induced by photoperiod in a tropical locust, *Nomadacris septemfasciata* (Serv.). *Ann. appl. Biol.* **50**, 600-603.
- NORRIS, M. J. (1965) The influence of constant and changing photoperiods on imaginal diapause in the red locust (*Nomadacris septemfasciata* Serv.). *J. Insect Physiol.* **11**, 1105-1119.
- NOVAK, V. J. A. (1966) *Insect Hormones*, 3rd edition. Methuen & Co. Ltd., London.
- NOVICKI, A. and WEEKS, J.C. (1996) The initiation of pre-ecdysis and ecdysis behaviours in larval *Manduca sexta*: the roles of the brain, terminal ganglion and eclosion hormone. *J. Exp. Biol.* **199**, 1757-1769.
- NOWOSIELSKI, J. W. and PATTON, R. L. (1963) Studies on circadian rhythms of the house cricket, *Gryllus domesticus* L. *J. Insect Physiol.* **9**, 401-410.
- NUMATA, H. (1992) Temporal variation in the photoperiodic induction and termination of adult diapause in the bean bug, *Riptortus clavatus*. *J. Insect Physiol.* **38**, 447-452.
- NUMATA, H. and HIDAKA, T. (1987) Photoreceptors for photoperiodism in the bean bug, *Riptortus clavatus*. *Rostrum* **38**, 571-580.
- NUMATA, H. and KOBAYASHI, S. (1994) Threshold and quantitative photoperiodic responses exist in an insect. *Experientia* **50**, 969-971.
- NUMATA, H. and NISIMURA, T. (2001) Circannual rhythm in *Anthrenus verbasci*: a re-evaluation of Blake's results. Proc. XXI Internat. Congress of Entomology, Iguassu Falls, Brazil.
- NUMATA, H., SAULICH, A.H. and VOLKOVICH, T.A. (1993) Photoperiodic responses of the linden bug, *Pyrrhocoris apterus* under conditions of constant temperature and under thermoperiodic conditions. *Zool. Sci.* **10**, 521-527.

- NUMATA, H., SHIGA, S. and MORITA, A. (1997) Photoperiodic receptors in arthropods. *Zool. Sci.* **14**, 187-197.
- ODHIAMBO, T. R. (1966) The metabolic effects of the corpus allatum hormone in the male desert locust. II. Spontaneous locomotor activity. *J. exp. Biol.* **45**, 51-63.
- OEHMKE, M. G. (1973) Lunar periodicity in flight activity of honey bees. *J. interdiscipl. Cycle Res.* **4**, 319-335.
- OHTAKI, T. (1966) On the delayed pupation of the flesh fly, *Sarcophaga peregrina* Robineau-Desvoidy. *Jap.J. Med. Sci.* **19**, 97-104.
- OHTAKI, T., MILKMAN, R. D. and WILLIAMS, C. M. (1968) Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **135**, 322-334.
- OKARINEN, A. and LUMME, J. (1979) Selection against photoperiodic reproductive diapause in *Drosophila littoralis*. *Hereditas* **90**, 119-125.
- OKADA, Y., TOMIOKA, K. and CHIBA, Y. (1991) Circadian phase-response curves for light in nymphal and adult crickets, *Gryllus bimaculatus*. *J. insect Physiol.* **37**, 583-590.
- OLDFIELD, G. N. (1970) Diapause and polymorphism in Californian populations of *Psylla pyri* (Homoptera: Psyllidae). *Ann. ent. Soc. Am.* **63**, 180-184.
- ONO, T., CHARLTON, R.E. and CARDE, R.T. (1990) Variability in pheromone composition and periodicity of pheromone titer in potato tuberworm moth, *Phthorimaea oeruclella* (Lepidoptera: Gelechiidae). *J. Chem. Ecology* **16**, 531-542.
- ORCHARD, I., DONLY, B.C., FUSE, M., LANGE, A.B., TOBE, S.S. and BENDENA, W.G. (1997) FMRF amide-related peptides in insects, with emphasis on the myosuppressins. *Neuropeptides in Development and Aging* **814**, 307-309.
- ORCHARD, I. and LANGE, A.B. (1988) The regulation of insect visceral muscle by octopamine. In: Boulton, A.A., Juorio, A.V. and Downe, R.G. (Eds.), *Trace amines*. Humana Press, Clifton, New Jersey, pp. 41-51.
- ORR, D.P.-Y. (1982) Genetic analysis of the circadian clock system of *Drosophila melanogaster*. PhD thesis. California Institute of Technology, Pasadena, CA, US.
- ORSHAN, L. and PENER, M. P. (1979a) Termination and reinduction of reproductive diapause by photoperiod and temperature in males of the grasshopper, *Oedipoda miniata*. *Physiol. Ent.* **4**, 55-61.
- ORSHAN, L. and PENER, M.P. (1979b) Repeated reversal of the reproductive diapause by photoperiod and temperature in males of the grasshopper, *Oedipoda miniata*. *Ent. Exp. Appl.* **25**, 219-226.
- OTTIGER, M., SOLLER, M., STOCKER, R.F. and KUBLI, E. (2000) Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J. Neurobiology* **44**, 57-71.
- OVERMEER, W.P.J., NELIS, H.J.C.F., DE LEENHEER, A.P., CALIS, J.N.M. and VEERMAN, A. (1989) Effect of diet on the photoperiodic induction of diapause in three species of predatory mite, *Amblyseius potentillae*, *A. cucumeris* and *Typhlodromus pyri*. *Exp. & Appl. Acarology* **7**, 281-287.
- PAARMANN, W. (1977) Propagation rhythm of subtropical and tropical Carabidae (Coleoptera) and its control by exogenous factors. In: *Advances in Invertebrate Reproduction*, vol. I (Ed. ADIYODI, K. G. and ADIYODI, R. G.). Peralam-Kenoth, Kerala, India. pp. 49-60.
- PADGHAM, D.E. (1981) hatching rhythms in the desert locust, *Schistocerca gregaria*. *Ohysiol. Entomol.* **6**, 191-198.
- PAGE, T. L. (1978) Interactions between bilaterally paired components of the cockroach circadian system. *J. comp. Physiol.* **124**, 225-236.
- PAGE, T.L. (1982) Transplantation of the cockroach circadian pacemaker. *Science* **216**, 73-75.
- PAGE, T.L. (1983a) Regeneration of the optic tracts and circadian pacemaker activity in the cockroach *Leucophaea maderae*. *J. comp. Physiol. A* **152**, 231-240.
- PAGE, T.L. (1983b) Effects of optic-tract regeneration on internal coupling of the circadian system of the cockroach. *J. comp. Physiol. A*, **153**, 353-363.
- PAGE, T.L. (1985) Circadian organization in cockroaches: effects of temperature cycles on locomotor activity. *J. Insect Physiol.* **31**, 235-242.
- PAGE, T.L. (1985) Clocks and circadian rhythms. In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Kerkut, G.A. and Gilbert, L.I. (eds) vol 6, Pergamon Press, Oxford, pp. 577-652.
- PAGE, T.L. (1987) Serotonin phase-shifts the circadian rhythm of locomotor activity in the cockroach. *J. Biol. Rhythms* **2**, 23-34.
- PAGE, T.L. (1988) Circadian organization and the representation of circadian information in the nervous system of invertebrates. In: *Advances in the Biosciences*, Vol. 73. Pp. 67-79. Pergamon Press plc.
- PAGE, T.L. (1990) Circadian rhythms of locomotor activity in cockroach nymphs. *J. Biol. Rhythms* **5**, 273-289.

- PAGE, T.L. (1991) Developmental manipulation of the circadian pacemaker in the cockroach: relationship between pacemaker period and response to light. *Physiol. Entomol.* **16**, 243-248.
- PAGE, T.L. and BARRETT, R.K. (1989) Light cycle effects on circadian pacemaker development II. Response to light. *J. comp. Physiol.* **165**, 51-59.
- PAGE, T. L. and BLOCK, G. D. (1980) Circadian rhythmicity in cockroaches: effects of early post-embryonic development and ageing. *Physiol. Ent.* **5**, 271-281.
- PAGE, T. L., CALDAROLA, P. C. and PITTENDRIGH, C. S. (1977) Mutual entrainment of bilaterally distributed circadian pacemakers. *Proc. Nat. Acad. Sci. U.S.A.* **74**, 1277-1281.
- PALMEN, E. (1955) Diel periodicity of pupal emergence in natural populations of some chironomids (Diptera). *Ann. Zool. Soc. Zool. Bot. Fennicae Vonamo*, **17**, 1-30.
- PARK, O. (1937) Studies in nocturnal ecology. Further analysis of activity in the beetle, *Passalus cornutus*, and description of audio-frequency recording apparatus. *J. Anim. Ecol.* **6**, 239-253.
- PARK, Y., ZITNAN, D., GILL, S.S. and ADAMS, M.E. (1999). Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. *FEBS Lett.* **463**, 133-138.
- PARKER, A. H. (1962) Studies on the diurnal rhythms of the house fly, *Musca domestica* L., in a dry tropical environment. *Acta Tropica*, **19**, 97-119.
- PARKER, J. R. (1930) Some effects of temperature and moisture upon *Melanoplus mexicanus* Saussure and *Camnula pellucida* Scudder (Orthoptera). *Bull. Univ. Montana Agric. Exp. Sta.* **223**, 132 pp.
- PARKER, M. W., HENDRICKS, S. B., BORTHWICK, H. A., and SCULLY, N. J. (1946) Action spectrum for the photoperiodic control of floral initiation of short-day plants. *Bot. Gaz.* **108**, 1-26.
- PARIS, O. H. and JENNER, C. E. (1959) Photoperiodic control of diapause in the pitcher-plant midge, *Metriocnemus knabi*. In *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. WITHROW, R. B.), pp. 601-624. Am. Ass. Adv. Sci., Washington.
- PAVLIDIS, T. (1968) Studies on biological clocks: a model for the circadian rhythms of nocturnal organisms. In: *Lectures on Mathematics in the Life Sciences*, vol. 1, Ed. GERSTENHABER, M. Amer. Math. Soc., Providence, R.I. pp. 88-112.
- PAVLIDIS, T. (1969) Populations of interacting oscillators and circadian rhythms. *J. theor. Biol.* **22**, 418-436.
- PAVLIDIS, T. (1971a) Populations of biochemical oscillators as circadian clocks. *J. theor. Biol.* **33**, 319-338.
- PAVLIDIS, T. (1971b) Mathematical models of circadian rhythms: their usefulness and their limitations. In *Biochronometry* (Ed. MENAKER, M.) pp. 110-116. National Academy of Sciences, Washington.
- PAVLIDIS, T. (1978a) Quantitative similarities between the behavior of coupled oscillators and circadian rhythms. *Bull. Math. Biol.* **40**, 675-692.
- PAVLIDIS, T. (1978b) What do mathematical models tell us about circadian clocks? *Bull. Math. Biol.* **40**, 625-635.
- PAVLIDIS, T. (1981) Mathematical models. In: *Handbook of Behavioral Neurobiology*, vol 4, *Biological Rhythms*. Ed: J. Aschoff. Pp 41-54. Plenum Press, New York.
- PAVLIDIS, T. and KAUZMANN, W. (1969) Toward a quantitative biochemical model for circadian oscillators. *Arch. Bioch. Biophys.* **132**, 338-348.
- PAVLIDIS, T., ZIMMERMAN, W.F. and OSBORN, J (1968) A mathematical model for temperature effects on circadian rhythms. *J. theor. Biol.* **18**, 210-221.
- PEARL, R. (1928) *The Rate of Living*. University of London Press.
- PEASE, R. W. (1962) Factors causing seasonal forms in *Ascia monuste* (Lepidoptera). *Science, Wash.* **137**, 987-988.
- PELC, D. and STEEL, C.G.H. (1997) Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotrophic hormone. *Gen. Comp. Endocrinol.* **108**, 358-365.
- PENER, M.P. and GREENFIELD, M.D. (1992) Variable length of reproductive diapause in males of the grasshopper *Anacridium aegyptium* and the effect of the corpora allata on sexual behaviour. In *Advances in Regulation of Insect Reproduction*, Eds: Bennetova, B., Gelbic, I. and Soldan, T. pp. 221-226.
- PENGELLEY, E. T. and FISHER, K. C. (1963) The effect of temperature and photoperiod on the yearly hibernating behavior of captive golden-mantled ground squirrels (*Citellus lateralis tescorum*). *Can. J. Zool.* **41**, 1103-1120.
- PEREZ, Y., VERDIER, M. and PENER, M. P. (1971) The effect of photoperiod on male sexual behaviour in a north adriatic strain of the migratory locust. *Entomologia exp.appl.* **14**, 245-250.
- PETERSON, D. M. and HAMNER, W. M. (1968) Photoperiodic control of diapause in the codling moth. *J. Insect Physiol.* **14**, 510-528.
- PETERSON, E. L. (1980a) Phase-resetting a mosquito circadian oscillator. I. Phase-resetting surface. *J. comp. Physiol.* **138**, 201-211.

- PETERSON, E. L. (1980b) A limit cycle interpretation of a mosquito circadian oscillator. *J. theoret. Biol.* **84**, 281-310.
- PETERSON, E. L. (1981) Dynamic response of a circadian pacemaker II. Recovery from light pulse perturbations. *Biol. Cybern.* **40**, 181-194.
- PETERSON, E. L. and JONES, M. D. R. (1979) Do circadian oscillations ever stop in constant light? *Nature, Lond.* **280**, 677-679.
- PETERSON, E. L. and SAUNDERS, D. S. (1980) The circadian eclosion rhythm in *Sarcophaga argyrostoma*: a limit cycle representation of the pacemaker. *J. theoret. Biol.* **86**, 265-277.
- PETRI, B. and STENGL, M. (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci.* **17**, 4087-4093.
- PETRI, B. and STENGL, M. (2001) Phase response curves of a molecular model oscillator: implications for mutual coupling of paired oscillators. *J. Biol. Rhythms* **16**, 125-141.
- PFLÜGER, W. and NEUMANN, D. (1971) Die Steuerung einer gezeitenparallelen Schlüpfrythmik nach dem Sanduhr-Prinzip. *Oecologia, Berl.* **7**, 262-266.
- PICIMBON, J.-F., BECARD, J.-M., SPENG, L., CLEMENT, J.-L. and GADENNE, C. (1995) Juvenile hormone stimulates pheromotropotropic brain factor release in the female black cutworm, *Agrotis ipsilon*. *J. Insect Physiol.* **41**, 377-382.
- PICIMBON, J.-F., GADENNE, C., BECARD, J.-M., CLEMENT, J.-L. and SRENG, L. (1997) Sex pheromone of the French black cutworm moth, *Agrotis ipsilon* (Lepidoptera: Noctuidae): Identification and regulation of a multicomponent blend. *J. Chem. Ecol.* **23**, 211-230.
- PITTENDRIGH, C. S. (1954) On temperature independence in the clock controlling emergence time in *Drosophila*. *Proc. Nat. Acad. Sci. U.S.A.* **40**, 1018-1029.
- PITTENDRIGH, C. S. (1958) Perspectives in the study of biological clocks. In *Perspectives in Marine Biology* (Ed. BUZZATI-TRAVERSO, A. A.), pp. 239-268. University of California Press.
- PITTENDRIGH, C. S. (1960) Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 159-184.
- PITTENDRIGH, C. S. (1961) On temporal organization in living systems. *Harvey Lectures, Ser.* **56**, 93-125.
- PITTENDRIGH, C. S. (1965) On the mechanism of entrainment of a circadian rhythm by light cycles. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 277-297. North-Holland, Amsterdam.
- PITTENDRIGH, C. S. (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. *Z. Pflanzenphysiol.* **54**, 275-307.
- PITTENDRIGH, C. S. (1967a) Circadian rhythms, space research and manned space flight. In *Life Sciences and Space Research*, pp. 122-134. North-Holland, Amsterdam.
- PITTENDRIGH, C. S. (1967b) Circadian systems I. The driving oscillation and its assay in *Drosophila pseudoobscura*. *Proc. Nat. Acad. Sci. U.S.A.* **58**, 1762-1767.
- PITTENDRIGH, C. S. (1972) Circadian surfaces and the diversity of possible roles of circadian organization in photoperiodic induction. *Nat. Acad. Sci. U.S.A.* **69**, 2734-2737.
- PITTENDRIGH, C. S. (1974) Circadian oscillations in cells and the circadian organization of multicellular systems. In *The Neurosciences Third Study Program* (Ed. SCHMITT, F. O. and WORDEN, F. G.), pp. 437-458. M.I.T. Press, Cambridge, Mass.
- PITTENDRIGH, C. S. (1981a) Circadian systems: entrainment. In: *Handbook of Behavioral neurobiology*, vol 4, *Biological Rhythms*. Ed: Aschoff, J. Plenum Press, New York and London, pp. 95-124.
- PITTENDRIGH, C. S. (1981b) Circadian organization and the photoperiodic phenomena. In *Biological Clocks in Reproductive Cycles* (Ed. B.K. Follett and D.E. Follett). John Wright, Bristol. pp. 1-35.
- PITTENDRIGH, C. S. (1993) Temporal organization: Reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.*, **55**, 17-54.
- PITTENDRIGH, C. S. and BRUCE, V. G. (1957) An oscillator model for biological clocks. In *Rhythmic and Synthetic Processes in Growth* (Ed. RUDNICK, D.), pp. 75-109. Princeton.
- PITTENDRIGH, C. S. and BRUCE, V. G. (1959) Daily rhythms as coupled oscillator systems and their relation to thermoperiodism and photoperiodism. In *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. WITHROW, R. B.), pp. 475-505. Am. Ass. Adv. Sci., Washington.
- PITTENDRIGH, C. S., BRUCE, V. G. and KAUS, P. (1958) On the significance of transients in daily rhythms. *Proc. Nat. Acad. Sci. U.S.A.* **44**, 965-973.
- PITTENDRIGH, C. S. and CALDAROLA, P. C. (1973) General homeostasis of the frequency of circadian oscillations. *Proc. Nat. Acad. Sci. U.S.A.* **70**, 2697-2701.
- PITTENDRIGH, C. S., CALDAROLA, P. C. and COSBEY, E. S. (1973) A differential effect of heavy water on temperature-dependent and temperature-compensated aspects of the circadian system of *Drosophila pseudoobscura*. *Proc. Nat. Acad. Sci. U.S.A.* **70**, 2037-2041.

- PITTENDRIGH, C. S. and DAAN, S. (1974) Circadian oscillations in rodents: a systematic increase of their frequency with age. *Science, Wash.* **186**, 548-550.
- PITTENDRIGH, C. S. and DAAN, S. (1976a) A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *J. comp. Physiol.* **106**, 223-252.
- PITTENDRIGH, C. S. and DAAN, S. (1976b) A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J. comp. Physiol.* **106**, 291-331.
- PITTENDRIGH, C. S. and DAAN, S. (1976c) A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. *J. comp. Physiol.* **106**, 333-355.
- PITTENDRIGH, C. S., EICHHORN, J. H., MINIS, D. H. and BRUCE, V. G. (1970) Circadian systems VI. Photoperiodic time measurement in *Pectinophora gossypiella*. *Proc. Nat. Acad. Sci. U.S.A.* **66**, 758-764.
- PITTENDRIGH, C.S., ELLIOT, T. and TAKAMURA, T. (1984) The circadian component in photoperiodic induction. In: *Photoperiodic Regulation of Insect and Molluscan Hormones. Ciba Foundation Symposium* **104**, 26-47.
- PITTENDRIGH, C.S., KYNER, W.T. and TAKAMURA, T. (1991) The amplitude of circadian oscillations: Temperature dependence, latitudinal clines, and the photoperiodic time measurement. *J. Biol. Rhythms* **6**, 299-313.
- PITTENDRIGH, C. S. and MINIS, D. H. (1964) The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Am. Nat.* **98**, 261-294.
- PITTENDRIGH, C. S. and MINIS, D. H. (1971) The photoperiodic time measurement in *Pectinophora gossypiella* and its relation to the circadian system in that species. In *Biochronometry* (Ed. MENAKER, M.), pp. 212-250. National Academy of Sciences, Washington.
- PITTENDRIGH, C. S. and MINIS, D. H. (1972) Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci. U.S.A.* **69**, 1537-1539.
- PITTENDRIGH, C. S. and SKOPIK, S. D. (1970) Circadian systems, V. The driving oscillation and the temporal sequence of development. *Proc. Nat. Acad. Sci. U.S.A.* **65**, 500-507.
- PITTENDRIGH, C.S. and TAKAMURA, T. (1987) temperature dependence and evolutionary adjustment of critical nightlength in insect photoperiodism. *Proc. Nat. Acad. Sci. USA* **84**, 7169-7173.
- PITTENDRIGH, C.S. and TAKAMURA, T. (1989) Latitudinal clines in the properties of a circadian pacemaker. *J. Biol. Rhythms* **4**, 217-235.
- PIVNICK, K.A. (1993) Response of males to female sex pheromone in the orange wheat blossom midge, *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae). *J. Chem. Ecology* **19**, 1677-1689.
- PLAUTZ, J.D., KANEKO, M., HALL, J.C. and KAY, S.A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. *Science* **278**, 1632-1635.
- POPE, M.M., GASTON, L.K. and BAKER, T.C. (1982) Composition, quantification and periodicity of sex pheromone gland volatiles from individual *Heliothis virescens* females. *J. Chem. Ecology* **8**, 1043-1056.
- POPHOF, B. (2000) Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J. Comp. Physiol. A* **186**, 307-313.
- PREDER, R. and ECKERT, M. (2000) Neurosecretion: peptidergic systems in insects. *Naturwiss.* **87**, 343-350.
- PRICE, J.L., BLAU, J., ROTHENFLUH, A., ABODEELY, M., KLOSS, B. and YOUNG, M.W. (1998) *double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**, 83-95.
- PROVOST, M. W. and LUM, P. T. M. (1967) The pupation rhythm in *Aedes taeniorhynchus* (Diptera: Culicidae). I. Introduction. *Ann. Ent. Soc. Am.* **60**, 138-149.
- PULLEN, A.S. (1996) Physiological relationships between insect diapause and cold tolerance: coevolution or coincidence? *Europ. J. Entomol.* **93**, 121-129.
- PYZA, E. and MEINERTZHAGEN, I.A. (1993) Daily and circadian rhythms of synaptic frequency in the first visual neuropile of the housefly's (*Musca domestica* L.) optic lobe. *Proc. R. Soc. Lond. B.* **254**, 97-105.
- PYZA, E. and MEINERTZHAGEN, I.A. (1995) Day/night size changes in lamina cells are influenced by the *period* gene in *Drosophila*. *Soc. Neurosci. Abstr.* **21**, 408.
- PYZA, E. and MEINERTZHAGEN, I.A. (1996) Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobes. *J. Comp. Physiol. A* **178**, 33-45.
- PYZA, E. and MEINERTZHAGEN, I.A. (1997) Neurites of *period*-expressing PDH cells in the optic lobe of the housefly exhibit circadian oscillations in morphology. *Eur. J. Neurosci.* **9**, 1784-1788.
- PYZA, E. and MEINERTZHAGEN, I.A. (1998) Neurotransmitters alter the numbers of synapses and organelles in the photoreceptor terminals of the housefly, *Musca domestica*. *J. Comp. Physiol. A* **183**, 719-727.
- PYZA, E. and MEINERTZHAGEN, I.A. (1999a) The role of clock genes and glial cells in expressing circadian rhythms in the fly's lamina. In *Neurobiology of Drosophila Abstracts*. Heberlein, U. and Keshishian, H., eds. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, p. 145.

- PYZA, E. and MEINERTZHAGEN, I.A. (1999b) Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. *J. Neurobiol.* **40**, 77-88.
- RAFAELI, A. (1994) Pheromonotropic stimulation of moth pheromone gland cultures *in vitro*. *Arch. Insect Biochem. Physiol.* **25**, 287-299.
- RAFAELI, A. and GILEADI, C. (1997) Neuroendocrine control of pheromone production in moths. *Invert Neurosci.* **3**, 223-229.
- RAFAELI, A., GILEADI, C., FAN, Y. and CAO, M. (1997) Physiological mechanisms of pheromonostatic responses: effects of adrenergic agonists and antagonists on moth (*Helicoverpa armigera*) pheromone biosynthesis. *J. Insect Physiol.* **43**, 261-269.
- RAFAELI, A., HIRSCH, J., SOROKER, V., KAMENSKY, B. and RAINA, A.K. (1991) Spatial and temporal oral distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa (Heliothis) armigera* using RIA and *in vitro* bioassay. *Arch. Insect Biochem. Physiol.* **18**, 119-129.
- RAFAELI, A. and SOROKER, V. (1989) Influence of diel rhythm and brain hormone on pheromone production in two Lepidopteran species. *J. Chem. Ecology* **15**, 447-456.
- RAFAELI, A., SOROKER, V., HIRSCH, J., KAMENSKY, B. and RAINA, A.K. (1993) The influence of photoperiod and age on the competence of pheromone glands and the distribution of immunoreactive PBAN in *Helicoverpa* spp. *Arch. Insect Biochem. Physiol.* **22**, 169-180.
- RAINA, A.K., JAFFE, H., KEMPE, T.G., KEIM, P., BLACHER, R.W., FALES, H.M., RILEY, C.T., KLUN, J.A., RIDGWAY, R.L. and HAYES, D.K. (1989) Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* **244**, 796-798.
- RAINA, A.K. and KLUM, J.A. (1984) Brain factor control of sex pheromone production in the female corn earworm moth. *Science* **225**, 531-533.
- RAINA, A.K. and MENN, J.J. (1987) Regulation of lepidoptera pheromone production. In: Prestwich, G.D. and Blomquist, G.J. (Eds), *Pheromone Biochemistry*. Academic Press, New York, pp. 159-174.
- RAMASWAMY, S.B. and CARDE, R.T. (1984) Rate of release of spruce budworm (*Choristoneura fumiferana*) pheromone from virgin females and synthetic lures. *J. Chem. Ecology* **10**, 1-8.
- RAMASWAMY, S.B., JURENKA, R.A., LINN, C.E. and ROELOFS, W.L. (1995) Evidence for the presence of a pheromonotropic factor in haemolymph and regulation of sex pheromone production in *Helicoverpa zea*. *J. Insect Physiol.* **41**, 501-508.
- RAMASWAMY, S.B., MBATA, G.N., COHEN, N.E., MOORE, A. and COX, N.M. (1994) Pheromonotropic and pheromonostatic activity in moths. *Arch. Insect Biochem. Physiol.* **25**, 301-315.
- RANKIN, M. A., CALDWELL, R. L. and DINGLE, H. (1972) An analysis of a circadian rhythm of oviposition in *Oncopeltus fasciatus*. *J. exp. Biol.* **56**, 353-359.
- RASENICK, M. M., NEUBERG, M. and BERRY, S. J. (1976) Brain cyclic AMP levels and the initiation of adult development in the cecropia silkworm. *J. Insect Physiol.* **22**, 1453-1456.
- RASENICK, M. M., NEUBERG, M. and BERRY, S. J. (1978) Cyclic nucleotide activation of the silkworm brain-cellular localization and further observations on the patterns of activation. *J. Insect Physiol.* **24**, 137-139.
- RAZUMOVA, A. P. (1978) Endogenous annual rhythm of the photoperiodic reaction in *Tetranychus crataegi* (Acarine: Tetranychidae). *Zool. Zh.* **57**, 530-539. (In Russian.)
- READ, D. C. (1969) Rearing the cabbage maggot with and without diapause. *Can. Ent.* **101**, 725-737.
- REICHLE, D. E., PALMER, J. D. and PARK, O. (1965) Persistent rhythmic locomotor activity in the cave cricket *Hadenocercus subterraneus* and its ecological significance. *Amer. Midl. Nat.* **74**, 57-66.
- REINHARDT, R. (1969) Über den Einfluss der Temperatur auf den Saisondimorphismus von *Araschnia levana* L. (Lepidopt. Nymphalidae) nach photoperiodischer Diapause-Induktion. *Zool. Jb. Physiol.* **75**, 41-75.
- REINHARDT, R. (1978) Untersuchungen zur Jahresrhythmik der Insektizidempfindlichkeit an der Stubenfliege *Musca domestica* L. *Zool. Jb. Physiol.* **8**, 200-204.
- REISCHIG, T. and STENGL, M. (1997) The accessory medulla is the presumptive circadian pacemaker in the cockroach *Leucophaea maderae*. *Verh. Dtsch Zool. Ges.* **89**-3, 45.
- REITER, P. and JONES, M. D. R. (1975) An eclosion timing mechanism in the mosquito *Anopheles gambiae*. *J. Ent. A.* **50**, 161-168.
- REITER, R.J. (1993) The melatonin rhythm: both a clock and a calendar. *Experientia* **49**, 654-664.
- REMMERT, H. (1961) Der Tagesgang im Strandanwurf und seine ökologische Bedeutung. *Verh. dt. Zool. Ges. Saarbrücken*, 1961, p. 438.
- REMMERT, H. (1962) *Der Schlupfrhythmus der Insekten*. Franz Steiner Verlag, Wiesbaden.
- RENCE, B.G. (1984) A comparison of light and temperature entrainment: evidence for a multioscillator system. *Physiol. Entomol.* **9**, 215-227.

- RENCE, B.G., LISY, M.T., GARVES, B.R. and QUINLAN, B.J. (1988) The role of ocelli in circadian singing rhythm of crickets. *Physiol. Entomol.* **13**, 201-212.
- RENCE, B. and LOHER, W. (1975) Arrhythmically singing crickets: thermoperiodic re-entrainment after bilobectomy. *Science, Wash.* **190**, 385-387.
- RENN, S.C.P., PARK, J.H., ROSBASH, M., HALL, J.C. and TAGHERT, P.H. (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**, 791-802.
- RENNER, M. (1955) Ein Transozeanversuch zum Zeitsinn der Honigbiene. *Naturwissenschaften*, **42**, 540-541.
- RENNER, M. (1957) Neue Versuche über den Zeitsinn der Honigbiene. *Z. vergl. Physiol.* **40**, 85-118.
- RENNER, M. (1959) Über ein weiteres Versetzungs-experiment zur Analyse des Zeitsinnes und der Sonnenorientierung der Honigbiene. *Z. vergl. Physiol.* **42**, 449-483.
- RENSING, L. (1961) Aktivitätsperiodik des Wasserläufers *Velia currens* F. *Z. vergl. Physiol.* **44**, 292-322.
- RENSING, L. (1964) Daily rhythmicity of corpus allatum and neurosecretory cells in *Drosophila melanogaster* (Meig.). *Science, Wash.* **144**, 1586-1587.
- RENSING, L. (1969) Die circadiane Rhythmik der Speicheldrüsen von *Drosophila* in vivo, in vitro, und unter dem Einfluss von Ecdyson. *J. Insect Physiol.* **15**, 2285-2303.
- RENSING, L. (1971) Hormonal control of circadian rhythms in *Drosophila*. In: Biochronometry. Menaker, M. (Ed.), National Academy of Sciences, Washington, D.C., pp. 527-540.
- RENSING, L., DORING, R., PROBECK, D., NAGEI, G. and RZEPKA, P. (1973) Circadian rhythms of transcription and possible controlling factors in rat liver and *Drosophila* salivary glands. *Int. J. Chronobiol.* **1**, 353-354.
- RENSING, L. and HARDELAND, R. (1967) Zur Wirkung der circadianen Rhythmik auf die Entwicklung von *Drosophila*. *J. Insect Physiol.* **13**, 1547-1568.
- RENSING, L., THACH, B. T. and BRUCE, V. G. (1965) Daily rhythms in the endocrine glands of *Drosophila* larvae. *Experientia*, **21**, 103-104.
- REVOL, J.F. and MARSCHESSAULT, R.H. (1993) *In vitro* chiral nematic ordering of chitin crystallites. *Intern. J. Biol. Macromolecules* **15**, 329-335.
- REYNOLDS, S.E. (1977) Control of cuticle extensibility in the wings of adult *Manduca* at the time of eclosion: Effects of eclosion hormone and bursicon. *J. Exp. Biol.* **70**, 27-39.
- REYNOLDS, S.E. (1980) Integration of behaviour and physiology in ecdysis. In: Berridge, M.J., Treherne, J.E. and Wigglesworth, V.B. (Eds.), *Advances in Insect Physiology*, vol. **15**. Academic Press, New York, pp 475-696.
- REYNOLDS, S.E. (1985) Hormonal control of cuticle mechanical properties. In: Kerkut, G.A. and Gilbert, L.I. (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. **8**. Pergamon Press, Oxford, pp 335-351.
- REYNOLDS, S.E. (1987) Endocrine timing signals that direct ecdysis, physiology and behaviour. In: Borkovec, A.B. and Gelman, D. (Eds.), *Insect Neurochemistry and Neurophysiology*, Human Press, Clifton, New Jersey, pp. 53-77.
- REYNOLDS, S.E., TAGHERT, P.H. and TRUMAN, J.W. (1979) Eclosion hormone and bursicon titres and the onset of hormonal responsiveness during the last day of adult development in *Manduca sexta*. *J. Exp. Biol.* **78**, 77-86.
- RIBA, G. (1976) Alimentation des larves de *Rhagium inquisitor* L. (Coleoptera, Cerambycidae) en conditions experimentales rythme prandial, transit intestinal, indices de consommation et de croissance. *Ann. Zool. Ecol. anim.* **8**, 499-511.
- RICHARD, D.S. and SAUNDERS, D.S. (1987) Prothoracic gland function in diapause and nondiapause *Sarcophaga argyrostoma* and *Calliphora vicina*. *J. Insect Physiol.* **33**, 385-392.
- RICHARD, D.S., SAUNDERS, D.S., EGAN, V.M. and THOMPSON, R.C.K. (1986) The timing of larval wandering and puparium formation in the flesh-fly, *Sarcophaga argyrostoma*. *Physiol. Entomol.* **11**, 53-60.
- RICHARD, D.S., WARREN, J.T., SAUNDERS, D.S. and GILBERT, L.I. (1987) Haemolymph ecdysteroid titres in diapause and nondiapause destined larvae and pupae of *Sarcophaga argyrostoma*. *J. Insect Physiol.* **33**, 115-122.
- RICHARD, D.S., WATKINS, N.L., SERAFIN, R.B. and GILBERT, L.I. (1998) Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol.* **44**, 637-644.
- RICHARDS, G. (1981) Insect hormones in development. *Biol. Rev.* **56**, 501-549.
- RICHTER, K. (2001) Daily changes in neuroendocrine control of moulting hormone secretion in the prothoracic gland of the cockroach, *Periplaneta americana* (L.). *J. Insect Physiol.* **47**, 333-338.
- RICHTER, K., PESCHKE, E. and PESCHKE, D. (1999) Effect of melatonin on the release of prothoracitropic hormone from the brain of *Periplaneta americana* (Blattodea: Blattidae). *Europ. J. Ent.* **96**, 341-345.

- RIDDIFORD, L.M. (1985) Hormone action at the cellular level. In: Kerkut, G.A. and Gilbert, L.I. (Eds.) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8. Pergamon Press, Oxford, pp. 37-84.
- RIDDIFORD, L. M. and TRUMAN, J. W. (1978) Biochemistry of insect hormones and insect growth regulators. In *Biochemistry of Insects*, pp. 308-357. Academic Press, New York.
- RIDDIFORD, L.M. and WILLIAMS, C.M. (1971) Role of corporal cardiaca in the behaviour of saturniid moths. *Biol. Bull.* **140**, 1-7.
- RIEDL, B. and CROFT, B.A. (1978) The effect of photoperiod and effective temperatures on the seasonal phenology of the codling moth (Lepidoptera: Tortricidae). *Can. Ent.* **110**, 455-470.
- RIEDL, H. and LOHER, W. (1980) Circadian control of oviposition in the codling moth, *Laspeyresia pomonella*, Lepidopter: Olethreutidae. *Ent. Exp. et Applic.* **27**, 38-49.
- RIEMANN, J.G. and GIEBULTOWICZ, J.M. (1991) Secretion in the upper vas deferens of the gypsy moth correlated with the circadian rhythm of sperm release from the testes. *J. Insect Physiol.* **37**, 53-62.
- RIEMANN, J.G. and GIEBULTOWICZ, J.M. (1992) Sperm maturation in the upper vas deferentia of the gypsy moth, *Lymantria dispar*. *Int. J. Insect Morph. Embryol.* **21**, 271-284.
- RIEMANN, J.G. and THORSON, B.J. (1971) Sperm maturation in the male and female genital tracts of *Anagasta kuehniella* (Lepidoptera: Pyralidae). *Int. J. Insect Morphol. Embryol.* **1**, 11-19.
- RIEMANN, J.G. and THORSON, B.J. (1976) Ultrastructure of the vasa deferentia of the Mediterranean flour moth. *J. Morph.* **149**, 483-506.
- RIEMANN, J.G., THORSON, B.J. and RUUD, R.L. (1974) Daily cycle of release of sperm from the testes of the Mediterranean flour moth. *J. Insect Physiol.* **20**, 195-207.
- RING, R. A. (1967) Maternal induction of diapause in the larvae of *Lucilia caesar* L. (Diptera, Calliphoridae). *J. exp. Biol.* **46**, 123-136.
- RITCHIE, M.G., HALSEY, E.J. and GLENSON, J.M. (1999) *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou and Hall cycles in *D. melanogaster* song. *Anim. Behav.* **58**, 649-657.
- RITCHIE, M.G. and KYRIACOU, C.P. (1994) Reproductive isolation and the *period* gene of *Drosophila*. *Molec. Ecol.* **3**, 595-599.
- RIVAULT, C. (1976) The role of the eyes and ocelli in the initiation of circadian activity rhythms in cockroaches. *Physiol. Ent.* **1**, 277-286.
- ROBERTS, B. and WARREN, M. H. (1975) Diapause in the Australian flesh-fly *Tricholioprocta impatiens* (Diptera: Sarcophagidae). *Austr. J. Zool.* **23**, 563-567.
- ROBERTS, B., HENRICH, V. and GILBERT, L.I. (1987) Effect of photoperiod on the timing of larval wandering in *Drosophila melanogaster*. *Physiol. Entomol.* **12**, 175-180.
- ROBERTS, R.M., NORTHOVER, J.M. and LEWIS, R.D. (1983) Circadian clock control of the eclosion rhythm of the brown blowfly, *Calliphora stygia* (Diptera Calliphoridae). *N.Z. Ent.* **7**, 424-431.
- ROBERTS, S. K. de F. (1956) 'Clock' controlled activity rhythm in the fruit fly. *Science, Wash.* **124**, 172.
- ROBERTS, S. K. de F. (1960) Circadian activity in cockroaches. I. The freerunning rhythm in steady-state. *J. cell. Comp. Physiol.* **55**, 99-110.
- ROBERTS, S. K. de F. (1962) Circadian activity in cockroaches. II. Entrainment and phase-shifting. *J. cell. Comp. Physiol.* **59**, 175-186.
- ROBERTS, S. K. de F. (1965a) Photoreception and entrainment of cockroach activity rhythms. *Science, Wash.* **148**, 958-959.
- ROBERTS, S. K. de F. (1965b) Significance of endocrines and central nervous system in circadian rhythms. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 198-213. North-Holland, Amsterdam.
- ROBERTS, S. K. de F. (1966) Circadian activity rhythms in cockroaches. III. The role of endocrine and neural factors. *J. Cell Physiol.* **67**, 473-486.
- ROBERTS, S. K. de F. (1974) Circadian rhythms in cockroaches. Effects of optic lobe lesions *J. comp. Physiol.* **88**, 21-30.
- ROBERTS, S. K. de F., SKOPIK, S. D. and DRISKILL, R. J. (1971) Circadian rhythms in cockroaches: does brain hormone mediate the locomotor cycle? In *Biochronometry* (Ed. MENAKER, M.), pp. 505-516. National Academy of Sciences, Washington.
- ROBINSON, A.S., HERFST, M. and VOSSELMAN, L. (1980) Genetic potential of *Delia antiqua* (Meigen) (Diptera: Anthomyiidae). Sensitivity to diapause interfering with a field-cage experiment using a homozygous chromosomal translocation. *Bull. Entomol. Res.* **70**, 103-111.
- ROBINSON, M.W., BAKER, P.S. and FINLAYSON, L.H. (1985) Influence of temperature changes on larviposition rhythm in the tsetse fly, *Glossina morsitans*. *Physiol. Entomol.* **10**, 215-220.

- ROCK, G.C. (1983) Thermoperiodic effects on the regulation of larval diapause in the tufted apple budmoth (Lepidoptera: Tortricidae). *Environmental Entomol.* **12**, 1500-1503.
- ROCKEY, S.J., MILLER, B.B. and DENLINGER, D.L. (1989) A diapause maternal effect in the flesh fly, *Sarcophaga bullata*: transfer of information from mother to progeny. *J. Insect Physiol.* **35**, 553-558.
- ROENNEBERG, T. and FOSTER, R.G. (1997) Twilight times: light and the circadian system. *Photochemistry and Photobiology* **66**, 549-561.
- ROENNEBERG, T. and MERROW, M. (1998) Molecular circadian oscillators: an alternative hypothesis. *J. Biol. Rhythms* **13**, 167-179.
- ROENNEBERG, T. and MERROW, M. (2001) Circadian systems – different levels of complexity. *Phil. Trans. R. Soc. Lond. B* **356**, 1687-1696.
- ROENNEBERG, T. and MORSE, D. (1993) two oscillators in one cell. *Nature* **362**, 362-364.
- ROSATO, E. and KYRIACOU, C.P. (2001) Flies, clocks and evolution. *Phil. Trans. R. Soc. Lond. B* **356**, 1769-1778.
- ROTH, R. L. and SOKOLOVE, P. G. (1975) Histological evidence for direct connections between the optic lobes of the cockroach *Leucophaea maderae*. *Brain Res.* **87**, 23-39.
- ROUBAUD, E. (1917) Observations biologiques sur *Nasonia brevicornis* Ashm., Chalcidide parasite des pupes des Muscides. Determinisme physiologique de l'instinct de ponte; adaptation a la lutte contre les glossines. *Bull. scient. Fr. Belg.* **1**, 425-439.
- ROUNDS, H. D. (1975) A lunar rhythm in the occurrence of blood borne factors in cockroaches, mice and men. *Comp. Biochem. Physiol. (C)*, **50**, 193-197.
- ROUNDS, H.D. (1981) Semi-lunar cyclicity of neurotransmitter-like substances in the CNS of *Periplaneta americana* (L.). *Comp. Biochem. Physiol.* **69C**, 293-299.
- ROUNDS, H.D. (1983) Lunar and seasonal variation in cardiac response to acetylcholine and noradrenaline. *Comp. Biochem. Physiol.* **74C**, 373-376.
- ROUNTREE, D.B. and NIJHOUT, H.F. (1995) Hormonal control of a seasonal polyphenism in *Precis coenia* (Lepidoptera: Nymphalidae). *J. Insect Physiol.* **41**, 987-992.
- ROWAN, W. (1926) On photoperiodism, reproductive periodicity and the annual migration of birds and certain fishes. *Proc. Boston Soc. nat. Hist.* **38**, 147-189.
- RUCKES, H. (1919) Notes on the male genital system in certain Lepidoptera. *Ann. Ent. Soc. Am.* **12**, 192-209.
- RUNTE, T. and WEBER, F. (1982) Synchronization of deposition of daily growth layers in the cuticle of the cockroach *Blaberus fuscus* by gating of moult. *Experientia* **38**, 1049-1051.
- RUOFF, P., VINSJEVIK, M., MONNERJAHN, C. and RENSING, L. (1999) The Goodwin oscillator: on the importance of degradation reactions in the circadian clock. *J. Biol. Rhythms* **14**, 469-479.
- RUTILA, J.E., SURJ, V., LE, M., SO, W.V., ROSBASH, M. and HALL, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell* **93**, 805-814.
- RYAN, R. B. (1965) Maternal influence on diapause in a parasitic insect, *Coeloides brunneri* (Vier.) (Hymenoptera: Braconidae). *J. Insect Physiol.* **11**, 1331-1336.
- SABROSKY, C. W., LARSON, I. and NABOURS, R. K. (1933) Experiments with light upon reproduction, growth and diapause in grouse locusts. *Trans. Kansas Acad. Sci.* **36**, 298-300.
- SACCA, G. (1964) Comparative bionomics in the genus *Musca*. *Ann. Rev. Ent.* **9**, 341-358.
- SAEZ, L. and YOUNG, M.W. (1988) In situ localization of the per clock protein during development of *Drosophila melanogaster*. *Molec. Cell. Biol.* **8**, 5378-5385.
- SAHOTA, T.S., RUTH, D.S., IBARAKI, A., FARRIS, S.H. and PEET, F.G. (1982) Diapause in the pharate adult stage of insect development. *Can. Ent.* **114**, 1179-1183.
- SAINT-PAUL, U. and ASCHOFF, J. (1978) Longevity among blowflies *Phormia terraenovae* R.D. kept in non-24-hour light-dark cycles. *J. comp. Physiol.* **127**, 191-195.
- SAKAI, T. and ISHIDA, N. (2001) Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natn. Acad. Sci. USA* **98**, 9221-9225.
- SAKAI, T. and MASAKI, S. (1965) Photoperiod as a factor causing seasonal forms in *Lycaena phlaeas daimio* Seitz. (Lepidoptera: Lycaenidae). *Kontyu*, **33**, 275-283.
- SAKAMOTO, K. and SHIMIZU, I. (1994) Photosensitivity in the circadian hatching rhythm of the carotenoid-depleted silkworm, *Bombyx mori*. *J. Biol. Rhythms* **9**, 61-70.
- SAKURAI, S. (1983) Temporal organization of endocrine events underlying larval-larval ecdysis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* **29**, 919-932.

- SAKURAI, S. (1984) Temporal organization of endocrine events underlying larval-pupal metamorphosis in the silkworm, *Bombyx mori*. *J. Insect Physiol.* **30**, 657-664.
- SAKURAI, S., KAYA, M. and SATAKE, S. (1998) Haemolymph ecdysteroid titre and ecdysteroid-dependent developmental events in the larval-pupal stadium of the silkworm, *Bombyx mori*: role of low ecdysteroid titre in larval-pupal metamorphosis and a reappraisal of the head critical period. *J. Insect Physiol.* **44**, 867-881.
- SALL, C., TSOUPRAS, G., KAPPLER, C., LAGEAUX, M., ZACHARY, D., LUU, B. and HOFFMANN, J.A., (1983) Maternal conjugated ecdysteroids during embryonic development in *Locusta migratoria*. [Journal?] **29**, 491-507.
- SANTSCHI, F. (1911) Observations et remarques critiques sur le mecanisme de l'orientation chez les fourmis. *Revue suisse Zool.* **19**, 303-338.
- SANTSCHI, F. (1913) A propos de l'orientation virtuelle chez les fourmis. *Bull. Soc. Hist. Nat. Afr. Nord.* **4-6**, 231-235.
- SAROV-BLAT, L., SO, W.V., LIU, L. and ROSBASH, M. (2000) The *Drosophila takeout* gene is a novel link between circadian rhythms and feeding behavior. *Cell* **101**, 647-656.
- SASAKI, M., JIBIKI, F. and HIROBE, T. (1984) Comparison of the daily rhythmic behaviour between wild and domestic silkmths, *Bombyx mandarina* and *Bombyx mori*. *Bull. Fac. Agric. Tamagawa University* **24**, 26-42.
- SASAKI, M., YAMAZAKI, S. and CHIBA, K. (1987) Brain photoreception in the calling rhythm of a noctuid moth, *Anadevidia peponis*: method of making eyeless moth and micro-irradiation of brain with fiber optics. *Bull. Fac. Agric. Tamagawa University* **27**, 81-92.
- SATO, Y., NAKAZAWA, Y., IMAI, K., KOMIYA, T., SAITO, H., SHIN, M., IKEDA, M., SAKAKIBURA, K., ISOBE, M. and YAMASHITA, O. (1992) A new diapause hormone molecule of the silkworm, *Bombyx mori*. *Proc. Jap. Acad. Sci. ser B* **68**, 75-79.
- SATO, Y., OGUCHI, M., MENJO, N., IMAI, K., SAITO, H., IKEDA, M., ISOBE, M. and YAMASHITA, O. (1993) Precursor polypeptide for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc. Nat. Acad. Sci. USA* **90**, 3251-3255.
- SAUER, K.P. (1984) The evolution of reproductive strategies as an adaptation to fluctuating temperatures. In *Advances in Invertebrate Reproduction 3* pp. 317-326. Ed: Engels, W., Clark, W.H. Jr., Fisher, A., Olive, P.J. and Went, D.F. Elsevier, Amsterdam.
- SAUMAN, I. and REPERT, S.M. (1996a) Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanism of *period* protein regulation. *Neuron* **17**, 889-900.
- SAUMAN, I. and REPERT, S.M. (1996b) Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkworm *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relation to circadian clock cells in central brain. *Devel. Biol.* **178**, 418-429.
- SAUMAN, I. and REPERT, S.M. (1998) Brain control of embryonic circadian rhythms in the silkworm *Antheraea pernyi*. *Neuron* **20**, 741-748.
- SAUMAN, I., TSAI, T., ROCA, A.L. and REPERT, S.M. (1996) Period protein is necessary for circadian control of egg hatching behavior in the silkworm *Antheraea pernyi*. *Neuron* **17**, 901-909.
- SAUNDERS, D. S. (1965a) Larval diapause induced by a maternally-operating photoperiod. *Nature, Lond.* **206**, 739-740.
- SAUNDERS, D. S. (1965b) Larval diapause of maternal origin: Induction of diapause in *Nasonia vitripennis* (Walk.) (Hymenoptera: Pteromalidae). *J. exp. Biol.* **42**, 495-508.
- SAUNDERS, D. S. (1966a) Larval diapause of maternal origin-II. The effect of photoperiod and temperature on *Nasonia vitripennis*. *J. Insect Physiol.* **12**, 569-581.
- SAUNDERS, D. S. (1966b) Larval diapause of maternal origin-III. The effect of host shortage on *Nasonia vitripennis*. *J. Insect Physiol.* **12**, 899-908.
- SAUNDERS, D. S. (1967) Time measurement in insect photoperiodism: reversal of a photoperiodic effect by chilling. *Science, Wash.* **156**, 1126-1127.
- SAUNDERS, D. S. (1968) Photoperiodism and time measurement in the parasitic wasp, *Nasonia vitripennis*. *J. Insect. Physiol.* **14**, 433-450.
- SAUNDERS, D. S. (1969) Diapause and photoperiodism in the parasitic wasp *Nasonia vitripennis*, with special reference to the nature of the photoperiodic clock. *Symp. Soc. exp. Biol.* **23**, 301-329.
- SAUNDERS, D. S. (1970) Circadian clock in insect photoperiodism. *Science, Wash.* **169**, 601-603.
- SAUNDERS, D. S. (1971) The temperature-compensated photoperiodic clock 'programming' development and pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. *J. Insect Physiol.* **17**, 801-812.
- SAUNDERS, D. S. (1972) Circadian control of larval growth rate in *Sarcophaga argyrostoma*. *Proc. Nat. Acad. Sci. U.S.A.* **69**, 2738-2740.

- SAUNDERS, D. S. (1973a) Thermoperiodic control of diapause in an insect: theory of internal coincidence. *Science, Wash.* **181**, 358-360.
- SAUNDERS, D. S. (1973b) The photoperiodic clock in the flesh-fly, *Sarcophaga argyrostoma*. *J. Insect Physiol.* **19**, 1941-1954.
- SAUNDERS, D. S. (1974) Evidence for 'dawn' and 'dusk' oscillators in the *Nasonia* photoperiodic clock. *J. Insect Physiol.* **20**, 77-88.
- SAUNDERS, D. S. (1975a) Spectral sensitivity and intensity thresholds in *Nasonia* photoperiodic clock. *Nature, Lond.* **233**, 732-734.
- SAUNDERS, D. S. (1975b) 'Skeleton' photoperiods and the control of diapause and development in the flesh-fly, *Sarcophaga argyrostoma*. *J. comp. Physiol.* **97**, 97-112.
- SAUNDERS, D. S. (1975c) Manipulation of the length of the sensitive period, and the induction of pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. *J. Ent. (A)*, **50**, 107-118.
- SAUNDERS, D. S. (1976) The circadian eclosion rhythm in *Sarcophaga argyrostoma*: some comparisons with the photoperiodic clock. *J. comp. Physiol.* **110**, 111-133.
- SAUNDERS, D. S. (1978a) An experimental and theoretical analysis of photoperiodic induction in the flesh-fly *Sarcophaga argyrostoma*. *J. comp. Physiol.* **124**, 75-95.
- SAUNDERS, D. S. (1978b) Internal and external coincidence and the apparent diversity of photoperiodic clocks in the insects. *J. comp. Physiol.* **127**, 197-207.
- SAUNDERS, D. S. (1979a) External coincidence and the photoinducible phase in the *Sarcophaga* photoperiodic clock. *J. comp. Physiol.* **132**, 179-189.
- SAUNDERS, D. S. (1979b) The circadian eclosion rhythm in *Sarcophaga argyrostoma*: delineation of the responsive period for entrainment. *Physiol. Ent.* **4**, 263-274.
- SAUNDERS, D. S. (1980a) Light as an entraining agent for biological clocks. In: *Animals and Environmental Fitness* (Ed. GILLES, R.), pp. 363-383. Pergamon Press, Oxford.
- SAUNDERS, D. S. (1980b) Some effects of constant temperature and photoperiod on the diapause response of the flesh-fly, *Sarcophaga argyrostoma*. *Physiol. Ent.* **5**, 191-198.
- SAUNDERS, D. S. (1981a) Insect photoperiodism: Entrainment within the circadian system as a basis for time measurement. In: *Biological Clocks in Seasonal Reproductive Cycles* (Ed FOLLETT, B. K.), pp. 67-81. J. Wright & Sons Ltd., Bristol.
- SAUNDERS, D. S. (1981b) Insect photoperiodism: the clock and the counter. *Physiol. Ent.* **6**, 99-116.
- SAUNDERS, D. S. (1981c) Insect photoperiodism. In: *Handbook of Behavioral Neurobiology*, vol 4, *Biological Rhythms*. Ed. J. Aschoff. Plenum Press, New York. Pp. 411-447.
- SAUNDERS, D. S. (1982a) *Insect Clocks*, 2nd edition. Pergamon Press, Oxford.
- SAUNDERS, D. S. (1982b) The effect of ultra-short photoperiods on the seasonal clock in *Sarcophaga argyrostoma*. *J. comp. Physiol.* **145**, 421-429.
- SAUNDERS, D. S. (1982c) Photoperiodic induction of pupal diapause in *Sarcophaga argyrostoma*: temperature effects on circadian resonance. *J. Insect Physiol.* **28**, 305-310.
- SAUNDERS, D. S. (1983) A diapause induction-termination asymmetry in the photoperiodic responses of the linden bug, *Pyrrhocoris apterus*, and an effect of near-critical photoperiods on development. *J. Insect Physiol.* **29**, 399-405.
- SAUNDERS, D. S. (1984) Photoperiodic time measurement in *Sarcophaga argyrostoma*: an attempt to use daily temperature cycles to distinguish external from internal coincidence. *J. comp. Physiol.* **154**, 789-794.
- SAUNDERS, D. S. (1984) Introduction: the links between 'wet' and 'dry' physiology. In: *Photoperiodic Regulation of Insect and Molluscan Hormones*, Ciba Foundation Symposium **104**, 2-6.
- SAUNDERS, D. S. (1986) Many circadian oscillators regulate developmental and behavioural events in the flesh fly *Sarcophaga argyrostoma*. *Chronobiol. Intern.* **3**, 71-83.
- SAUNDERS, D. S. (1987) Maternal influence on the incidence and duration of larval diapause in *Calliphora vicina*. *Physiol. Entomol.* **12**, 331-338.
- SAUNDERS, D. S. (1987) Insect photoperiodism: the linden bug, *Pyrrhocoris apterus*, a species that measures daylength rather than nightlength. *Experientia* **43**, 935-937.
- SAUNDERS, D. S. (1990) The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: is the *period* gene causally involved in photoperiodic time measurement? *J. Biol. Rhythms* **5**, 315-331.
- SAUNDERS, D. S. (1992) The photoperiodic clock and "counter" in *Sarcophaga argyrostoma*: experimental evidence consistent with "external coincidence" in insect photoperiodism. *J. comp. Physiol.* **170**, 121-127.
- SAUNDERS, D. S. (1997) Under-sized larvae from short-day adults of the blow fly, *Calliphora vicina*, side-step the diapause programme. *Physiol. Entomol.* **22**, 249-255.

- SAUNDERS, D.S. (1998) Developmental processes in insects: circadian rhythms and photoperiodism in the blow fly, *Calliphora vicina*. Chapter 13 in: *Biological Rhythms and Photoperiodism in Plants*. Eds P.J. Lumsden and A. Millar. Bios Scientific Publishers, Oxford. Pp. 211-229.
- SAUNDERS, D.S. (2000) Arthropoda – Insecta: Diapause. In: *Reproductive Biology of Invertebrates*, Vol X, Part B, Chapter 5, pp. 145-184. Ed. A. Dorn; series eds. K.G. and R.G. Adiyodi. John Wiley & Sons Ltd.
- SAUNDERS, D.S. (2000) Larval diapause duration and fat metabolism in three geographical strains of the blow fly, *Calliphora vicina*. *J. Insect Physiol.* **46**, 509-517.
- SAUNDERS, D.S. (2001) Geographical strains and selection for the diapause trait. In: *Insect Timing: Circadian Rhythms to Seasonality*. Eds: D.L. Denlinger, J.M. Giebultowicz and D.S. Saunders. Elsevier Science. pp 113-121.
- SAUNDERS, D.S. and BRADLEY, H. (1984) Long-night summation and programming of pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. In *Photoperiodic Regulation of Insect and Molluscan Hormones*, Ciba Foundation Symposium **104**, 65-89.
- SAUNDERS, D.S. and CYMBOROWSKI, B. (1996) Removal of optic lobes of adult blow flies (*Calliphora vicina*) leaves photoperiodic induction of larval diapause intact. *J. Insect Physiol.* **42**, 807-811.
- SAUNDERS, D.S. and GILBERT, L.I. (1990) Regulation of ovarian diapause in the fruit fly *Drosophila melanogaster* by photoperiod at moderately low temperature. *J. Insect Physiol.* **36**, 195-200.
- SAUNDERS, D.S., GILLANDERS, S.W. and LEWIS, R.D. (1994) Light-pulse phase response curves for the locomotor activity rhythm in period mutants of *Drosophila melanogaster*. *J. Insect Physiol.* **40**, 957-968.
- SAUNDERS, D.S. and HAYWARD, S.A.L. (1998) Geographical and diapause-related cold tolerance in the blow fly, *Calliphora vicina*. *J. Insect Physiol.* **44**, 541-551.
- SAUNDERS, D.S., HENRICH, V.C. and GILBERT, L.I. (1989) Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. USA* **86**, 3748-3752.
- SAUNDERS, D.S. and HONG, S.-F. (2000) Effects of temperature and temperature-steps on circadian locomotor rhythmicity in the blow fly, *Calliphora vicina*. *J. Insect Physiol.* **46**, 289-295.
- SAUNDERS, D.S. and LEWIS, R.D. (1987a) A damped circadian oscillator model of an insect photoperiodic clock. II. Simulations of the shapes of the photoperiodic response curves. *J. theoret. Biol.* **128**, 61-71.
- SAUNDERS, D.S. and LEWIS, R.D. (1987b) A damped circadian oscillator model of an insect photoperiodic clock. III. Circadian and "hourglass" responses. *J. theoret. Biol.* **128**, 73-85.
- SAUNDERS, D.S. and LEWIS, R.D. (1988) The photoperiodic clock and counter mechanism in two species of flies: Evidence for damped circadian oscillators in time measurement. *J. comp. Physiol.* **A 163**, 365-371.
- SAUNDERS, D.S., RICHARD, D.S., APPLEBAUM, S.W., MA, M and GILBERT, L.I. (1990) Photoperiodic diapause in *Drosophila melanogaster* involves a block to the juvenile hormone regulation of ovarian maturation. *Gen. Comp. Endocrin.* **79**, 174-184.
- SAUNDERS, D. S. and SUTTON, D. (1969) Circadian rhythms in the insect photoperiodic clock. *Nature, Lond.* **221**, 559-561.
- SAUNDERS, D. S., SUTTON, D. and JARVIS, R. A. (1970) The effect of host species on diapause induction in *Nasonia vitripennis*. *J. Insect Physiol.* **16**, 405-416.
- SAUNDERS, D. S. and THOMSON, E. J. (1977) 'Strong' phase response curve for the rhythm of locomotor activity in a cockroach (*Nauphoeta cinerea*). *Nature, Lond.* **270**, 242-243.
- SAUNDERS, D.S., WHEELER, I. and KERR, A. (1999) Survival and reproduction of small blow flies (*Calliphora vicina*) produced in severely overcrowded short day larval cultures. *Eur. J. Entomol.* **96**, 19-22.
- SAUNDERS, J. L. and KNOKE, J. K. (1968) Circadian emergence rhythm of a tropical scolytid, *Xyleborus ferrugineus*. *Ann. ent. Soc. Am.* **61**, 587-590.
- SAWIN, E.P., DOWSE, H.B., HAMBLIN-COYLE, M.J., HALL, J.C. and SOKOLOWSKI, M.B. (1994) A lack of locomotor activity rhythms in *Drosophila melanogaster* larvae (Diptera: Drosophilidae). *J. Insect Behav.* **7**, 349-362.
- SAWIN-MCCORMACK, E.P., SOKOLOWSKI, M.B. and CAMPOS, A.R. (1995) Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. *J. Neurogenet.* **10**, 119-135.
- SAWYER, L.A., HENNESSEY, M.J., PEIXOTO, A.A., ROSATO, E., PARKINSON, H., COSTA, R. and KYRIACOU, C.P. (1997) Natural variation in *Drosophila* clock gene and temperature compensation. *Science* **278**, 2117-2120.
- SCHAL, C. and CARDE, R.T. (1986) Effects of temperature and light on calling in the tiger moth *Holomelina lamae* (Freeman) (Lepidoptera: Arctiidae). *Physiol. Entomol.* **11**, 75-87.
- SCHARRER, B. and SCHARRER, E. (1944) Neurosecretion, VI. A comparison between the intercerebralis-cardiacum-allatum system of insects and the hypothalamo-hypophyseal system of vertebrates. *Biol. Bull.* **87**, 242-251.

- SCHMID, H. and ENGELMANN, W. (1987) Effects of Li^+ , Rb^+ and tetraethylammonium chloride on the locomotor activity rhythm of *Musca domestica*. *J. interdiscipl. Cycle Res.* **18**, 83-102.
- SCHNEIDERMAN, H. A. and HORWITZ, J. (1958) The induction and termination of facultative diapause in the chalcid wasps *Mormoniella vitripennis* (Walker) and *Tritoneptis klugii* (Ratzeburg). *J. exp. Biol.* **35**, 520-551.
- SCHULTE, G. (1976) Tidal rhythms in feeding and defecation of a terrestrial mite (Oribatei: Ameronothridae). *Mar. Biol.* **37**, 265-277.
- SCHURMANN, F.-W. (1987) The architecture of the mushroom bodies and related neuropils in the insect brain. In: Gupta, A.P. (ed) *Arthropod Brain*, Wiley-Liss, New York, pp. 231-264.
- SCHWAB, R.G. (1971) Circannian testicular periodicity in the European starling in the absence of photoperiodic change. In *Biochronometry* (Ed. MENAKER, M.), pp. 428-447. National Academy of Sciences, Washington.
- SCHWARTZ, L.M. and TRUMAN, J.W. (1983) Hormonal control of rates of metamorphic development in the tobacco hornworm *Manduca sexta*. *Devel. Biol.* **99**, 103-114.
- SCHWEIGER, H.-G., HARTWIG, R. and SCHWEIGER, M. (1986) Cellular aspects of circadian rhythms. *Journal Cell Science Supplement* **4**, 181-200.
- SCHWEMMLE, B. (1960) Thermoperiodic effects and circadian rhythms in flowering of plants. *Cold Spring Harb. Symp. Quant. Biol.* **25**, 239-243.
- SCOTT, W. N. (1936) An experimental analysis of the factors governing the hour of emergence of adult insects from their pupae. *Trans. R. ent. Soc. Lond.* **85**, 303-329.
- SEFIANI, M. (1987) Regulation of egg laying and *in vitro* contractions in *Gryllus bimaculatus*. *J. Insect Physiol.* **33**, 215-222.
- SEHGAL, A., PRICE, J.L., MAN, B. and YOUNG, M.W. (1994) Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science* **263**, 1603-1606.
- SEHGAL, A., ROTHENFLUH-HILFKE, A., HUNTER-ENSOR, M., CHEN, Y., MYERS, M.P. and YOUNG, M.W. (1995) Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. *Science* **270**, 808-810.
- SHAKHBAZOV, V. G. (1961) The reaction of the length of daylight and the light receptor of the pupa of the Chinese oak silkworm *Antheraea pernyi* G. *Dokl. Akad. Nauk SSSR*, **140**, 249-252. (in Russian.)
- SHAPIRO, A. M. (1973) Photoperiodic control of seasonal polymorphism in *Pieris occidentalis* Reakirt (Lepidoptera: Pieridae). *Wasman J. Biol.* **31**, 291-299.
- SHAPIRO, A. M. (1975a) Developmental and phenotypic responses to photoperiod in uni- and bivoltine *Pieris napi* in California. *Trans. R. ent. Soc. Lond.* **127**, 65-71.
- SHAPIRO, A.M. (1975b) Photoperiodic control of development and phenotype in a subarctic population of *Pieris occidentalis* (Lepidoptera: Pieridae). *Can. Ent.* **107**, 775-779.
- SHAPIRO, A. M. (1977) Phenotypic induction in *Peris napi* L.: role of temperature and photoperiod in a coastal California population. *Ecol. Ent.* **2**, 217-224.
- SHARMA, M. L., LARRIVEE, J. M. and THERIAULT, L. M. (1975) Séquences de descendance des formes sexuées chez le puceron du pois, *Acyrtosiphon pisum* (Homoptera: Aphididae) en relation avec la durée des photopériodes. *Can. Ent.* **107**, 1063-1067.
- SHAW, P.J., CIRELLI, C., GREENSPAN, R.J. and TOMONI, G. (2000) Correlates of sleep and waking in *Drosophila melanogaster*. *Science* **287**, 1834-1837.
- SHEARER, P.W., JONES, V.P. and RIEDL, H. (1995) Diel periodicity and circadian control of oviposition by *Cryptophlebia illepidia* (Lepidoptera: Tortricidae). *Environ. Entomol.* **24**, 1229-1233.
- SHEEBA, V., CHANDRASHEKARAN, M.K., JOSHI, A. and SHARMA, V.K. (2001) Evidence for endogenous control of oviposition in individuals of *Drosophila melanogaster* reared in an aperiodic environment for several hundred generations. In press.
- SHEEBA, V., SHARMA, V.K., CHANDRASHEKARAN, M.K., and JOSHI, A. (1999a) Effect of different light regimes on pre-adult fitness in *Drosophila melanogaster* populations reared in constant light for over six hundred generations. *Biol. Rhythm Res.* **30**, 424-433.
- SHEEBA, V., SHARMA, V.K., CHANDRASHEKARAN, M.K., and JOSHI, A. (1999b) Persistence of eclosion rhythm after 600 generations in an aperiodic environment. *Naturwissenschaften* **86**, 448-449.
- SHIGA, S. and NUMATA, H. (1996) Effects of compound eye removal on the photoperiodic response of the band-legged ground cricket, *Pteronemobius nigrofasciatus*. *J. comp. Physiol. A* **179**, 625-633.
- SHIGA, S. and NUMATA, H. (1997) Induction of reproductive diapause via perception of photoperiod through the compound eyes in the adult blow fly, *Protophormia terraenovae*. *J. comp. Physiol. A* **181**, 35-40.
- SHIGA, S. and NUMATA, H. (2000) The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blow fly, *Protophormia terraenovae*. *Naturwiss.* **87**, 125-128.

- SHIGA, S., NUMATA, H. and YOSHIOKA, E. (1999) Localization of the photoreceptor and pacemaker for the circadian activity rhythm in the band-legged ground cricket, *Dianemobius nigrofasciatus*. *Zool Sci.* **16**, 193-201.
- SHIGA, S., TOYODA, I. and NUMATA, H. (2000) Neurons projecting to the retrocerebral complex of the adult blow fly, *Protophormia terraenovae*. *Cell Tissue Res.* **299**, 427-439.
- SHIMADA, K. (1985) Reduction in the critical number of short days for pupal diapause in *Papilio machaon* with precocious metamorphosis. *J. Insect Physiol.* **31**, 683-688.
- SHIMIZU, I. (1982) Photoperiodic induction in the silkworm, *Bombyx mori*, reared on artificial diet: evidence for extraretinal photoreception. *J. Insect Physiol.* **28**, 841-846.
- SHIMIZU, I. and HASEGAWA, K. (1988) Photoperiodic induction of diapause in the silkworm, *Bombyx mori*: location of the photoreceptor using a chemiluminescent paint. *Physiol. Entomol.* **13**, 81-88.
- SHIMIZU, I. and KATO, M. (1984) Carotenoid functions in photoperiodic induction in the silkworm, *Bombyx mori*. *Photobiochem. Photobiophys.* **7**, 47-52.
- SHIMIZU, I. and MIURA, K. (1987) Circadian clock controlling the eclosion rhythm of the silkworm *Bombyx mori*: its characteristics and dynamics. *Mem. Fac. Sci. Kyoto Univ. (ser Biol)* **12**, 135-156.
- SHIMIZU, T. and MASAKI, S. (1997) Daily hatching time in nemobiine crickets. *Jap. J. Ent.* **65**, 335-342.
- SHIOMI, K., ISHIDA, Y., IKEDA, M., SATO, Y., SAITO, H., IMAI, K., ISOBE, M. and YAMASHITA, O. (1994) Induction of non-diapause eggs by injection of anti-diapause hormone rabbit serum into the diapause type of silkworm, *Bombyx mori*. *J. Insect Physiol.* **40**, 693-699.
- SHIRAI, Y., AIZONO, Y., IWASAKI, T., YANAGIDA, A., MORI, H., SUMIDA, M. and MATSUBARA, F. (1993) Prothoracicotropic hormone is released five times in the 5th-larval instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **39**, 83-88.
- SHOREY, H.H. (1966) The biology of *Trichoplusia ni*. IV. Environmental control of mating. *Ann. Ent. Soc. Amer.* **59**, 502-506.
- SIEBER, R. and BENZ, G. (1977) Juvenile hormone in larval diapause of the codling moth, *Laspeyresia pomonella* L. (Lepidoptera, Tortricidae). *Experientia* **33**, 1598-1599.
- SIEBER, R. and BENZ, G. (1980) The hormonal regulation of the larval diapause in the codling moth, *Laspeyresia pomonella* (Lep. Tortricidae). *J. Insect Physiol.* **26**, 213-218.
- SIEGMUND, L.W. and KORGE, G. (2001) Innervation of the ring gland of *Drosophila*. *J. Comp. Neurology* **431**, 481-491.
- SIEW, Y. C. (1965a) The endocrine control of adult reproductive diapause in the chrysomelid beetle, *Galeruca tanacetii* (L.). I. *J. Insect Physiol.* **11**, 1-10.
- SIEW, Y. C. (1965b) The endocrine control of adult reproductive diapause in the chrysomelid beetle, *Galeruca tanacetii* (L.). II. *J. Insect Physiol.* **11**, 463-479.
- SIEW, Y. C. (1965c) The endocrine control of adult reproductive diapause in the chrysomelid beetle, *Galeruca tanacetii* (L.). III. *J. Insect Physiol.* **11**, 973-981.
- SIMMONS, L.W. and GWYNNE, D.T. (1993) Reproductive investment in bushcrickets: the allocation of male and female nutrients to offspring. *Proceedings of the Royal Society of London B* **252**, 1-5.
- SIWICKI, K.K., EASTMAN, C., PETERSEN, G., ROSBASH, M. and HALL, J.C. (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythm changes in the visual system. *Neuron* **1**, 141-150.
- SKOPIK, S. D. and BOWEN, M. F. (1976) Insect photoperiodism: An hourglass measures photoperiodic time in *Ostrinia nubilalis*. *J. comp. Physiol.* **111**, 249-259.
- SKOPIK, S. D. and PITTENDRIGH, C. S. (1967) Circadian systems, II. The oscillation in the individual *Drosophila* pupa; its independence of developmental stage. *Proc. Nat. Acad. Sci. U.S.A.* **58**, 1862-1869.
- SKOPIK, S. D. and TAKEDA, M. (1980) Circadian control of oviposition activity in *Ostrinia nubilalis*. *Am. J. Physiol.* **239**, R259-264.
- SKOPIK, S.D. and TAKEDA, M. (1986) Photoperiodic control of diapause induction and termination in *Ostrinia nubilalis*: Two different clocks? *J. Biol. Rhythms* **1**, 137-144.
- SKOPIK, S.D. and TAKEDA, M. (1987) Diapause induction and termination: North-south strain differences in *ostrinia nubilalis*. *J. Biol. Rhythms* **2**, 13-22.
- SKOPIK, S. D., TAKEDA, M. and HOLYOKE, C. W. (1981). A critical examination of the dual dual system theory in *Ostrinia nubilalis*. *Am. J. Physiol.* in press.
- SLAMA, K. (1964) Hormonal control of respiratory metabolism during growth, reproduction and diapause in female adults of *Pyrrhocoris apterus* L. *J. Insect Physiol.* **10**, 283-304.
- SLAMA, K. (1980) Homeostatic function of ecdysteroids in ecdysis and oviposition. *Acta Ent. Bohem.* **77**, 145-168.
- SMIETANKO, A. and ENGELMANN, W. (1989) Splitting of circadian activity rhythm of *Musca domestica* flies

- with azadirachtin. *J. interdiscipl. Cycle Res.* **20**, 71-79.
- SMITH, D.S. (1963) The organization and innervation of the luminescent organ in a firefly, *Photuris pennsylvanica* (Coleoptera). *J. Cell Biol.* **16**, 323-359.
- SMITH, P.H. (1983) Circadian control of spontaneous flight activity in the blowfly, *Lucilia cuprina*. *Physiol. Entomol.* **8**, 73-82.
- SMITH, P.H. (1985) Responsiveness to light of the circadian clock controlling eclosion in the blowfly, *Lucilia cuprina*. *Physiol. Entomol.* **10**, 323-336.
- SMITH, P.H. (1987) naturally occurring arrhythmicity in eclosion and activity in *Lucilia cuprina*: its genetic basis. *Physiol. Ent.* **12**, 99-107.
- SMITH, P.H., DALLWITZ, R., WARDHAUGH, K.G., VOGT, W.G. and WOODBURN, T.L. (1981) Timing of larval exodus from sheep and carrion in the sheep blowfly, *Lucilia cuprina*. *Ent. Exp. & appl.* **30**, 157-162.
- SMITH, R.F. and KONOPKA, R.J. (1981) Circadian clock phenotypes of chromosome aberrations with a breakpoint at the *per* locus. *Molec. Gen. Genet.* **183**, 243-251.
- SMITH, W.A. (1993) Second messengers and the action of prothoracicotropic hormone in *Manduca sexta*. *Amer. Zool.* **33**, 330-339.
- SO, W.V., SAROV-BLAT, L., KOTARSKI, C.K., McDONALD, M.J., ALLADA, R., and ROSBASH, M. (2000) *takeout*, a novel *Drosophila* gene under circadian clock transcriptional regulation. *Molec. Cell. Biol.* **20**, 6935-6944.
- SOKOLOVE, P. G. (1975) Locomotory and stridulatory circadian rhythms in the cricket, *Teleogryllus commodus*. *J. Insect Physiol.* **21**, 537-558.
- SOKOLOVE, P.G. and BUSHILL, W.N. (1978) The chi square periodogram: its utility for analysis of circadian rhythms. *J. Theoret. Biol.* **72**, 131-160.
- SOKOLOVE, P.G. and LOHER, W. (1975) Role of eyes, optic lobes and pars intercerebralis in locomotor and stridulatory circadian rhythms of *Teleogryllus commodus*. *J. Insect Physiol.* **21**, 785-799.
- SOLLER, M., BOWNES, M. and KUBLI, E. (1997) Mating and sex peptide stimulate the accumulation of yolk in the oocytes of *Drosophila melanogaster*. *Eur. J. Biochem.* **243**, 732-738.
- SOLLER, M., BOWNES, M. and KUBLI, E. (1999) Control of oocyte maturation in sexually mature *Drosophila* females. *Devel. Biol.* **208**, 337-351.
- SOMME, L. (1961) On the overwintering of house flies (*Musca domestica* L.) and stable flies (*Stomoxys calcitrans* (L.)) in Norway. *Norsk. Entomol. Tidsskr.* **11**, 191-223.
- SOUTHWICK, E.E. and MORITZ, R.F.A. (1987) Social synchronization of circadian rhythms of metabolism in honeybees (*Apis mellifera*). *Physiol. Entomol.* **12**, 209-212.
- SOUTHWOOD, T. R. E. (1962) Migration of terrestrial arthropods in relation to habit. *Biol. Rev.* **37**, 171-214.
- SOWER, L.L., SHOREY, H.H. and GASTON, L.K. (1970) Sex pheromones of noctuid moths, XXI. Light-dark cycle regulation and light inhibition of the sex pheromone release by females of *Trichoplusia ni*. *Ann. Ent. Soc. Am.* **63**, 1090-1092.
- SPANGLER, H. G. (1972) Daily activity rhythms of individual worker and drone honey bees. *Ann. ent. Soc. Am.* **65**, 1073-1076.
- SPIETH, H.R. and SAUER, K.P. (1991) Quantitative measurement of photoperiods and its significance for the induction of diapause in *Pieris brassicae* (Lepidoptera, Pieridae). *J. Insect Physiol.* **37**, 231-238.
- STANEWSKY, R., KANEKO, M., EMERY, P., BERETTA, B., WAGER-SMITH, K., KAY, S.A., ROSBASH, M. and HALL, J.C. (1998) The *cry^b* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell* **95**, 681-692.
- STANLEY-SAMUELSON, D.W. (1994) Prostaglandins and related eicosanoids in insects. *Adv. Insect Physiol.* **24**, 115-212.
- STAY, B., TOBE, S.S. and BENDENA, W.G. (1994) Allatostatins: Identification, primary structures, functions and distribution. *Adv. Insect Physiol.* **25**, 267-237.
- STEBBINS, R. C. (1963) Activity changes in the striped plateau lizard with evidence on influence of the parietal eye. *Copeia*, **1963**, 681-691.
- STEEL, C. G. H. (1975) A neuroendocrine feedback mechanism in the insect moulting cycle. *Nature*, **253**, 267-269.
- STEEL, C.G.H. and AMPLEFORD, E.J. (1984) Circadian control of haemolymph ecdysteroid titres and the ecdysis rhythm in *Rhodnius prolixus*. In: Porter, R. and Collins, G.M. (Eds.). *Photoperiodic Regulation of Insect and Molluscan Hormones*, Pitman, London, pp. 150-162.
- STEEL, C.G.H. and DAVEY, K.G. (1985) Integration of the insect endocrine system. In: Kerkut, G.A. and Gilbert, L.I. (Eds.) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 8, Pergamon Press, Oxford, pp. 1-35.

- STEEL, C. G. H. and LEES, A. D. (1977) The role of neurosecretion in the photoperiodic control of polymorphism in the aphid *Megoura viciae*. *J. exp. Biol.* **67**, 117-135.
- STEEL, C.G.H. and NSEIRI, S. (2002) Localization of neurons expressing prothoracicotrophic hormone- and bombyxin-like peptides in the brain of the insect, *Rhodnius prolixus*: Immunohistochemical and surgical studies. *Gen. Comp. Endocrinol.* (Submitted).
- STEEL, C.G.H. and VAFOPOULOU, X. (1989) Ecdysteroid titre profiles during growth and development of arthropods. In: Koolman, J. (ed). *Ecdysone*, Georg Thieme Verlag, Stuttgart, pp. 221-231.
- STEEL, C.G.H. and VAFOPOULOU, X. (1990) Circadian timing mechanisms in the endocrine system controlling growth and development in the insect, *Rhodnius prolixus* (Hemiptera). In: Loughton, B.G. and Saleudin, A.S.M., (Eds.). *Neurobiology and Endocrinology of Selected Invertebrates*, Captus University Publications, pp.11-26.
- STEEL, C.G.H. and VAFOPOULOU, X. (1999) A tango of hormone rhythms directs insect development: circadian regulation of prothoracicotrophic hormone and ecdysteroids. In: Roubos, E.W., Bonga, S.E.W., Vaudry, H. and DeLoof, A., (Eds.). *Shaker*, The Netherlands, pp. 94-97.
- STEEL, C.G.H. and VAFOPOULOU, X. (2001) Regulation of rhythmic steroidogenesis by light and neuro-peptide inputs during development in the insect *Rhodnius prolixus*. In: Goos, H.J.Th., Rastogi, R.K., Vaudry, H. and Pierantoni, R. (eds), *Perspectives in Comparative Endocrinology: Unity and Diversity*. Monduzzi Editore, Bologna, Italy, pp. 301-307.
- STENGL, M. and HOMBERG, U. (1994) Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J. comp. Physiol. A* **175**, 203-213.
- STOFFOLANO, J. G. and MATTHYSSE, J. G. (1967) Influence of photoperiod and temperature on diapause in the face fly, *Musca autumnalis* (Diptera: Muscidae). *Ann. ent. Soc. Am.* **60**, 1242-1246.
- STOUT, J., ATKINS, G. and ZACHARIUS, D. (1991) Regulation of cricket phonotaxis through hormonal control of the threshold of an identified auditory neuron. *J. Comp. Physiol. A* **169**, 765-772.
- STOUT, J., HAO, J., KIM, P., MBUNGU, D., BRONSERT, M., SLIKKERS, S., MAIER, J., KIM, D., BACCHUS, K. and ATKINS, G. (1998) Regulation of the phonotactic threshold of the female cricket, *Acheta domestica*: juvenile hormone III, allatectomy, L1 auditory neuron thresholds and environmental factors. *J. Comp. Physiol. A* **182**, 635-645.
- STROSS, R. G. and HILL, J. C. (1968) Photoperiod control of winter diapause in the fresh water crustacean, *Daphnia*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **134**, 176-198.
- STRUMWASSER, F. (1965) The demonstration and manipulation of a circadian rhythm in a single neuron. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 442-462. North-Holland, Amsterdam.
- SUGIKI, T. and MASAKI, S. (1972) Photoperiodic control of larval and pupal development in *Spilarctia imparilis* Butler (Lepidoptera: Arctiidae). *Kontyu*, **40**, 269-278.
- SUTER, R. B. and RAWSON, K. S. (1968) Circadian activity rhythm of the deer mouse, *Peromyscus*: Effect of deuterium oxide. *Science, Wash.* **160**, 1011-1014.
- SUZUKI, K., MINAGAWA, T., KUMAGAI, T., NAYA, S-I., ENDO, Y. OSANAI, M. and KUWANO, E. (1990) Control mechanism of diapause of the pharate first instar larvae of the silkworm *Antheraea yamamai*. *J. Insect Physiol.* **36**, 855-860.
- SUZUKI, K., NAKAMURA, T., YANBE, T., KURIHARA, M. and Kuwano, E. (1993) termination of diapause in pharate first-instar larvae of the gypsy moth *Lymantria dispar japonica* by an imidazole derivative KK-42. *J. Insect Physiol.* **39**, 107-110.
- SUZUKI, T. (1981) Effect of photoperiod on male egg production by foundresses of *Polistes chinensis antennalis* Perez (Hymenoptera, Vespidae). *Jap. J. Ecol.* **31**, 347-351.
- SWEENEY, B. M. (1969) *Rhythmic Phenomena in Plants*. Academic Press, New York and London.
- SWEENEY, B.M. (1974) A physiological model for circadian rhythms derived from the *Acetabularia* rhythm paradoxes. *Int. J. Chronobiol.* **2**, 25-33.
- SWEENEY, B.M. and HASTINGS, J. W. (1960) Effects of temperature on diurnal rhythms. *Cold Spr. Harb. Symp. Quant. Biol.* **25**, 87-104.
- SYBCEV, M.A., STAMENOVA, M.B. and BORISOVA, Y.A. (1986) Effect of the time of the day, age of insects and pheromone dose on the sexual reaction of *Musca domestica* (Dipter; Muscidae). *Ekologiya (Sofia)* **1**:62-67.
- SZOLLOSI, A. (1982) Relationships between germ and somatic cells in the testes of locusts and moths. In: King, R.C. and Akai, H. (eds), *Insect Ultrastructure*, Plenum Press, New York, pp. 32-60.
- TAKEDA, M. (1978) Photoperiodic time measurement and seasonal adaptation of the South-western corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Pyralidae). Ph.D. thesis, University of Missouri-Columbia, U.S.A.

- TAKEDA, M. (1983) Ontogeny of the circadian system governing ecdysial rhythms in a holometabolous insect, *Diatraea grandiosella* (Pyralidae). *Physiol. Entomol.* **8**, 321-331.
- TAKEDA, M. (1986) A circadian clock controlling cricket photoperiodism: A resonance effect. *J. Insect Physiol.* **32**, 557-560.
- TAKEDA, M., ENDO, Y., OHNISHI, H. and ICHIHARA (1999) Photoperiodic system in physiological reality. *Ent. Sci.* **2**, 567-574.
- TAKEDA, M. and MASAKI, S. (1976) Photoperiodic control of larval development in *Plodia interpunctella*. Proc. joint U.S./Japanese Seminar on Stored product Insects, Manhattan, Kansas, pp. 186-201. Kansas State Univ.
- TAKEDA, M. and MASAKI, S. (1979) Asymmetric perception of twilight affecting diapause induction by the fall webworm, *Hyphantria cunea*. *Ent. exp. appl.* **25**, 317-327.
- TAKEDA, M. and SKOPIK, S.D. (1985) Geographic variation in the circadian system controlling photoperiodism in *Ostrinia nubilalis*. *J. comp. Physiol. A* **156**, 653-658.
- TAKEDA, M. and SKOPIK, S.D. (1997) Photoperiodic time measurement and related physiological mechanisms in insects and mites. *Ann. Rev. Entomol.* **42**, 323-349.
- TAKIMOTO, A. and HAMNER, K. C. (1964) Effect of temperature and preconditioning on photoperiodic response of *Pharbitis nil*. *Pl. Physiol.* **39**, 1024-1030.
- TANAKA, S. (1976) Wing polymorphism, egg production and adult longevity in *Pteronemobius taprobanensis* Walker (Orthoptera: Gryllidae). *Kontyu*, **44**, 327-333.
- TANAKA, S., MATSUKA, M. and SAKAI, T. (1976) Effect of change in photoperiod on wing form in *Pteronemobius taprobanensis* Walker (Orthoptera: Gryllidae). *Appl. Ent. Zool.* **11**, 27-32.
- TANAKA, S., DENLINGER, D.L. and WOLDA, H. (1987a) daylength and humidity as environmental cues for diapause termination in a tropical beetle. *Physiol. Entomol.* **12**, 213-224.
- TANAKA, S., DENLINGER, D.L. and WOLDA, H. (1988) Seasonal changes in the photoperiodic response regulating diapause in a tropical beetle, *Stenotarsus rotundus*. *J. Insect Physiol.* **34**, 1135-1142.
- TANAKA, S., HAKOMORI, T. and HASEGAWA, E. (1993) Effects of daylength and hopper density on reproductive traits in a Japanese population of the migratory locust, *Locusta migratoria* L. *J. Insect Physiol.* **39**, 571-580.
- TANAKA, S., WOLDA, H. and DENLINGER, D.L. (1987b) Seasonality and its physiological regulation in three neotropical insect taxa from Barro Colorado island, Panama. *Insect Sci. Applic.* **8**, 507-514.
- TANAKA, Y. (1944). Effect of daylength on hibernation of the chinese oak silkworm. *Jap. J. Agr. Hort.* **19** (9). (in Japanese.)
- TANAKA, Y. (1950a) Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar silkworm. I. *J. Seric. Sci. Japan*, **19**, 358-371. (In Japanese.)
- TANAKA, Y. (1950b) Studies on hibernation with special reference to photoperiodicity and the breeding of the Chinese Tussar silkworm - II. *J. Seric. Sci. Japan*, **19**, 429-A46. (In Japanese.)
- TANAKA, Y. (1950c) Studies on hibernation with special reference to photoperiodicity and and breeding of the Chinese Tussar silkworm - III. *J. Seric. Sci. Japan*, **19**, 580-590. (In Japanese.)
- TANAKA, Y. (1951) Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar silkworm-V. *J. Seric. Sci. Japan*, **20**, 132-138. (In Japanese.)
- TANG, J.D., CHARLTON, R.E., CARDE, R.T. and YIN, C.M. (1992) Diel periodicity and influence of age and mating on sex pheromone titre in gypsy moth *Limantria dispar* (L.). *J. Chem. Ecology* **18**, 749-760.
- TAUBER, E. and KYRIACOU, C.P. (2001) Insect photoperiodism and circadian clocks: models and mechanisms. *J. Biol. Rhythms* **16**, 392-391.
- TAUBER, M. J. and TAUBER, C. A. (1970) Photoperiodic induction and termination of diapause in an insect: response to changing daylengths. *Science, Wash.* **167**, 170.
- TAUBER, M. J. and TAUBER, C. A. (1972) Geographic variation in critical photoperiod and in diapause intensity of *Chrysopa carnea* (Neuroptera). *J. Insect Physiol.* **18**, 25-29.
- TAUBER, M. J. and TAUBER, C. A. (1973a) Nutritional and photoperiodic control of the seasonal reproductive cycle in *Chrysopa mohave* (Neuroptera). *J. Insect Physiol.* **19**, 729-736.
- TAUBER, M. J. and TAUBER, C. A. (1973b) Quantitative responses to daylength during diapause in insects. *Nature, Lond.* **244**, 296-297.
- TAUBER, M. J. and TAUBER, C. A. (1976a) Developmental requirements of the univoltine species *Chrysopa downsi*: photoperiodic stimuli and sensitive stage. *J. Insect Physiol.* **22**, 331-335.
- TAUBER, M. J. and TAUBER, C. A. (1976b) Environmental control of univoltinism and evolution in an insect species. *Can. J. Zool.* **54**, 260-265.
- TAUBER, M. J., TAUBER, C. A. and DENYS, C. J. (1970) Adult diapause in *Chrysopa carnea*: photoperiodic control of duration and colour. *J. Insect Physiol.* **16**, 949-955.

- TAUBER, M. J., TAUBER, C. A. and MASAKI, S. (1986) *Seasonal Adaptations of Insects*. Oxford University Press, Oxford.
- TAWATA, M and ICHIKAWA, T. (2001) Circadian firing of neurosecretory cells releasing pheromonomotropic neuropeptides in the silkworm, *Bombyx mori*. *Zool. Sci.* **18**, 645-649.
- TAYLOR, B. (1969) Geographical range and circadian rhythms. *Nature, Lond.* **222**, 296-297.
- TAYLOR, B. and JONES, M. D. R. (1969) The circadian rhythm of flight activity in the mosquito *Aedes aegypti* (L.): the phase-setting effects of light-on and light-off. *J. exp. Biol.* **51**, 59-70.
- TAYLOR, F. and KARBAN, R. (1986) *The Evolution of Insect Life Cycles*. Springer-Verlag. 287 pp.
- TAYLOR, L. R. and KALMUS, H. (1954) Dawn and dusk flight of *Drosophila subobscura*. *Nature, Lond.* **174**, 221.
- TAYLOR, W., KRASNOW, R., DUNLAP, J.C., BRODA, H. and HASTINGS, J.W. (1982) Critical pulses of anisomycin drive the circadian oscillator in *Gonyaulax* towards its singularity. *J. Comp. Physiol.* **148**, 11-25.
- TEAL, P.E.A., DAVIS, N.T., MEREDITH, J.A., CHRISTENSEN, T.A. and HILDEBRAND, J.G. (1999) Role of the ventral nerve cord and terminal abdominal ganglion in the regulation of sex pheromone production in the tobacco budworm (Lepidoptera: Noctuidae). *Ann. Ent. Soc. Am.* **92**, 891-901.
- TEAL, P.E.A., MEREDITH, J.A. and GOMEZ, S.Y. (1999) Isolation and identification of terpenoid sex pheromone components from extracts of haemolymph of males of the Caribbean fruit fly. *Arch. Insect Biochem. Physiol.* **42**, 225-232.
- TEAL, P.E.A., TUMLINSON, J.H. and OBERLANDER, H. (1989) Neural regulation of sex pheromone biosynthesis in *Heliothis* moths. *Proc. Nat. Acad. Sci., USA* **86**, 2488-2492.
- TERRY, K.L. and STEEL, C.G.H. (2002) Confocal analysis of PERIOD- and TIMELESS-immunoreactivity in the brain and prothoracic glands of *Rhodnius prolixus*: Localization of potential circadian oscillators in the neuroendocrine system. *J. Comp. Neurology* (Submitted).
- THIBOUT, E. (1980) Evolution and role of apyrene sperm cells in lepidopterans: their activation and denaturation in the leek moth *Acrolepiopsis assectella*. In: Clark, W.H. and Adams, T.S. (Eds), *Advances in Invertebrate Reproduction*, Elsevier, Amsterdam, pp. 231-242.
- THIELE, H-U. (1966) Einflüsse der Photoperiode auf die Diapause von carabiden. *Z. angew. Ent.* **58**, 143-149.
- THIELE, H-U. (1969) The control of larval hibernation and adult aestivation in the carabid beetles *Nebria brevicollis* F. and *Patrobus atrorufus*. *Oecologia, Berl.* **2**, 347-361.
- THIELE, H-U. (1971) Die Steuerung der Jahresrhythmik von Carabiden durch exogene und endogene Faktoren. *Zool. Jb. (Syst)*, **98**, 341-371.
- THIELE, H-U. (1973) Remarks about Mansingh's and Müller's classifications of dormancies in insects. *Can. Ent.* **105**, 925-928.
- THIELE, H-U. (1975) Interactions between photoperiodism and temperature with respect to the control of dormancy in the adult stage of *Pterostichus oblongopunctatus* F. (Col. Carabidae). I. Experiments on gonad maturation under different climatic conditions in the laboratory. *Oecologia, Berl.* **19**, 39-47.
- THIELE, H-U. (1977a) Measurement of daylength as a basis for photoperiodism and annual periodicity in the carabid beetle, *Pterostichus nigrita* F. *Oecologia, Berl.* **30**, 331-348.
- THIELE, H-U. (1977b) Differences in measurement of daylength and photoperiodism in two stocks from sub-arctic and temperate climates in the carabid beetle, *Pterostichus nigrita* F. *Oecologia, Berl.* **30**, 349-365.
- THIELE, H-U. and KONEN, H. (1975) Interactions between photoperiodism and temperature with respect to the control of dormancy in the adult stage of *Pterostichus oblongopunctatus* F. (Col., Carabidae). II. The development of the reproductive potential during the winter months in the field. *Oecologia, Berl.* **19**, 339-343.
- THOMSON, E. (1976) A study of circadian locomotor activity rhythms of the cockroach *Nauphoeta cinerea* (Oliver). B.Sc. thesis, University of Edinburgh.
- THOMPSON, K.J. (1986a) Oviposition digging in the grasshopper. I. Functional anatomy and the motor program. *J. Exp. Biology* **122**, 387-411.
- THOMPSON, K.J. (1986b) Oviposition digging in the grasshopper. II. Descending neural control. *J. Exp. Biol.* **122**, 413-425.
- THOMPSON, K.G. and SIEGLER, M.V.S. (1993) Development of segment specificity in identified lineages of the grasshopper CNS. *J. Neuroscience* **13**, 3309-2218.
- THORSON, B.J. and RIEMANN, J.G. (1977) Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J. Insect Physiol.* **23**, 1189-1197.
- THORSON, B.J. and RIEMANN, J.G. (1982) Effects of 20-hydroxyecdysone on sperm release from the testes of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller). *J. Insect Physiol.* **28**, 1013-1019.
- TILDEN, A.R., ANDERSON, W.J. and HUTCHISON, V.H. (1994) Melatonin in two species of damselfly, *Ishnura verticalis* and *Enallagma civile*. *J. Insect Physiol.* **40**, 775-780.

- TILLMAN, J.A., SEYBOLD, S.J., JURENKA, R.A. and BLOMQUIST, G.J. (1999) Insect pheromones - an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol.* **29**, 481-514.
- TIMMINS, G.S., ROBB, F.J., WIMOT, C.M., JACKSON, S.K. and SWARTZ, H.M. (2001) Firefly flashing is controlled by gating oxygen to light-emitting cells. *J. Exp. Biol.* **204**, 2795-2801.
- TOMA, D.P., BLOCK, G., MOORE, D., and ROBINSON, G.E. (2000) Changes in *period* mRNA levels in the brain and division of labor in honey bee colonies. *Proc. Nat. Acad. Sci. US* **97**, 6914-6919.
- TOMIOKA, K. (1985) Residual circadian rhythmicity after bilateral lamina-medulla removal or optic stalk transection in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* **31**, 653-657.
- TOMIOKA, K., AGUI, N. and BOLLENBACHER, W.E. (1995) Electrical properties of the cerebral prothoracicotrophic hormone cells in diapausing and nondiapausing pupae of the tobacco hornworm, *Manduca sexta*. *Zool. Sci.* **12**, 165-173.
- TOMIOKA, K. and CHIBA, Y. (1984) *Zool. Sci.* **1**, 375-382.
- TOMIOKA, K. and CHIBA, Y. (1986) Circadian rhythm in the neurally isolated lamina-medulla complex of the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* **32**, 747-755.
- TOMIOKA, K. and CHIBA, Y. (1989) Photoperiodic entrainment of locomotor activity in crickets (*Gryllus bimaculatus*) lacking the optic lobe pacemaker. *J. Insect Physiol.* **35**, 827-835.
- TOMIOKA, K., OKUDA, Y. and CHIBA, Y. (1990) Distribution of circadian photoreceptors in the compound eye of the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms* **5**, 303-313.
- TOMIOKA, K., SAKAMOTO, M., HARUI, Y., MATSUMOTO, N. and MATSUMOTO, A. (1998) Light and temperature cooperate to regulate the circadian locomotor rhythm of wild type and *period* mutants of *Drosophila melanogaster*. *J. Insect Physiol.* **44**, 587-596.
- TOMIOKA, K., UWAZUMI, K. and MATSUMOTO, N. (1997) Light cycles given during development affect free-running period of circadian locomotor rhythm of *period* mutants of *Drosophila melanogaster*. *J. Insect Physiol.* **43**, 297-305.
- TOMIOKA, K., WAKATSUKI, T., SHIMONO, K. and CHIBA, Y. (1991) Circadian control of hatching in the cricket, *Gryllus bimaculatus*. *J. Insect Physiol.* **37**, 365-371.
- TOMIOKA, K. and YUKIZANE, M. (1997) A specific area of the compound eye in the cricket *Gryllus bimaculatus* sends photic information to the circadian pacemaker in the contralateral optic lobe. *J. comp. Physiol.* **180**, 63-70.
- TOSINI, G., BERTOLUCCI, C. and FOA, A. (2001) The circadian system of reptiles: a multioscillatory and multiphotoreceptive system. *Physiology and Behavior* **72**, 461-471.
- TOUHARA, K. and PRESTWICH, G.D. (1992) Binding site mapping of a photoaffinity-labelled juvenile hormone binding protein. *Biochem. Biophys. Res. Commun.* **182**, 466-473.
- TOUHARA, K., LERRO, K.A., BONNING, B.C., HAMMOCK, B.D. and PRESTWICH, G.D. (1993) Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry* **32**, 2068-2075.
- TREHERNE, J. E. (1977) Free-running activity rhythm in the natural environment. *Nature*, **269**, 796-797.
- TREHERNE, J. E. and FOSTER, W. A. (1977) Diel activity of an intertidal beetle *Dicheirotrichus gustavi* Crotch. *J. anim. Ecol.* **46**, 127-138.
- TRIMMER, B.A., APRILLE, J.R., DUDZINSKI, D.M., LAGACE, C.J., LEWIS, S.M., MICHEL, T., QAZI, S. and ZAYAS, R.M. (2001) Nitric oxide and the control of firefly flashing. *Science* **292**, 2486-2488.
- TRUMAN, J. W. (1970) The eclosion hormone: its release by the brain, and its action on the central nervous system of silkworms. *Am. Zool.* **10**, 511-512.
- TRUMAN, J. W. (1971a) Hour-glass behavior of the circadian clock controlling eclosion of the silkworm *Antheraea pernyi*. *Proc. Nat. Acad. Sci. U.S.A.* **68**, 595-599.
- TRUMAN, J. W. (1971b) The role of the brain in the ecdysis rhythm of silkworms: comparison with the photoperiodic termination of diapause. In *Biochronometry* (Ed. MENAKER, M.), pp. 483-504. National Academy of Sciences, Washington.
- TRUMAN, J. W. (1971c) Physiology of insect ecdysis I. The eclosion behavior of Saturniid moths and its hormonal release. *J. exp. Biol.* **54**, 805-814.
- TRUMAN, J. W. (1971d) Circadian rhythms and physiology with special reference to neuroendocrine processes in insects. *Proc. Int. Symp. Circadian Rhythmicity* (Wageningen, 1971) pp. 111-135.
- TRUMAN, J. W. (1972a) Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. exp. Biol.* **57**, 805-820.
- TRUMAN, J. W. (1972b) Physiology of insect rhythms. II. The silkworm brain as the location of the biological clock controlling eclosion. *J. comp. Physiol.* **81**, 99-114.
- TRUMAN, J. W. (1973) Temperature sensitive programming of the silkworm flight clock: a mechanism for adapting to the seasons. *Science, Wash.* **182**, 727-729.

- TRUMAN, J. W. (1974) Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J. comp. Physiol.* **95**, 281-296.
- TRUMAN, J. W. (1976) Extraretinal photoreception in insects. *Photochem. Photobiol.* **23**, 215-225.
- TRUMAN, J.W. (1978a) Rhythmic control over endocrine activity in insects. In: Gaillard, P.J. and Boer, H.H. (Eds), *Comparative Endocrinology*, Elsevier, North Holland Biomedical Press, pp. 123-136.
- TRUMAN, J.W. (1978b) Hormonal release of stereotyped motor programmes from the isolated nervous system of the cecropia silkmoth. *J. exp. Biol.* **74**, 151-173.
- TRUMAN, J.W. (1985) Hormonal control of ecdysis. In: Kerkut, G.A. and Gilbert, L.I. (Eds), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol 8. Pergamon Press, Oxford, pp. 413-440.
- TRUMAN, J.W. (1990) Neuroendocrine control of ecdysis. In: Ohnishi, E. and Ishizaki, H. (Eds), *Moulting and Metamorphosis*. Springer-Verlag, Berlin, pp. 67-82.
- TRUMAN, J.W. (1992) The eclosion hormone of insects. *Prog. Brain Res.* **92**, 361-374.
- TRUMAN, J.W. and COPENHAVER, P.F. (1989) The larval eclosion hormone neurones in *Manduca sexta*: identification of the brain-protodeal neurosecretory system. *J. Insect Biol.* **147**, 457-470.
- TRUMAN, J.W. and MORTON, D.B. (1990) The eclosion hormone system: an example of coordination of endocrine activity during the moulting cycle of insects. In: Eppler, A., Scane, C.G. and Stetson, M.H. (Eds), *Progress in Comparative Endocrinology*. Wiley-Liss, New York, pp. 300-308.
- TRUMAN, J. W. and RIDDIFORD, L. M. (1970) Neuroendocrine control of ecdysis in silk-moths. *Science, Wash.* **167**, 1624-1626.
- TRUMAN, J.W. and RIDDIFORD, L.M. (1974a) Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* **60**, 371-382.
- TRUMAN, J.W. and RIDDIFORD, L.M. (1974b) Hormonal mechanisms underlying insect behaviour. *Adv. Insect Physiol.* **10**, 197-352.
- TRUMAN, J.W., HORODYSKI, F.M., NEWES, R.S. and RIDDIFORD, L.M. (1991) Eclosion hormone: from genes to behaviour. In: Menn, J.J., Kelly, T.J. and Masler, E.P. (Eds), *Insect Neuropeptides: Chemistry, Biology and Action*. A.C.S. Brooks, Washington, pp. 95-99.
- TRUMAN, J.W., ROUNTREE, D.B., REISS, S.E. and SCHWARTZ, L.H. (1983) Ecdysteroids regulate the release and action of eclosion hormone in the tobacco hornworm, *Manduca sexta* (L.). *J. Insect Physiol.* **29**, 895-900.
- TRUMAN, J. W. and SOKOLOVE, P. G. (1972) Silkmoth eclosion: hormonal triggering of a centrally programmed pattern of behavior. *Science, Wash.* **175**, 1491-1493.
- TRUMAN, J.W., TAGHERT, P.H., COPENHAVER, P.F., TUBLITZ, N.J. and SCHWARTZ, L.M. (1981) Eclosion hormone may control all ecdyses in insects. *Nature* **291**, 70-71.
- TSCHERNYSCHEV, W. B. and AFONINA, V. M. (1975) Biorhythm disturbances and the life-span of some insects. *Zh. obshch. Biol.* **36**, 859-862. (In Russian.)
- TSITSIPIS, J. A. and MITTLER, T. E. (1977a) Influence of daylength on the production of parthenogenetic and sexual forms of *Aphis fabae* at 17.5°C. *Ent. exp. appl.* **21**, 163-173.
- TSITSIPIS, J. A. and MITTLER, T. E. (1977b) Influence of temperature and daylength on the production of males, by *Aphis fabae*. *Ent. exp. appl.* **21**, 229-237.
- TSUMARAKI, J., ISHIGURO, J. and YAMANAKA, A. (1999) Effects of photoperiod and temperature on seasonal morph development and diapause egg oviposition in a bivoltine race (Daizo) of the silkmoth, *Bombyx mori*. *J. Insect Physiol.* **45**, 101-106.
- TWEEDY, D. G. and STEPHEN, W. P. (1970) Light-refractive emergence rhythm in the leaf-cutter bee, *Megachile rotundata* (F.) (Hy., Apoidea). *Experientia* **26**, 377-379.
- TYCHSEN, P. H. and FLETCHER, B. S. (1971) Studies on the rhythm of mating in the Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* **17**, 2139-2156.
- TYSHCHENKO, V. P. (1966) Two-oscillatory model of the physiological mechanism of insect photoperiodic reaction. *Zhur. obshch. Biol.* **27**, 209-222. (In Russian.)
- TYSHCHENKO, V. P., GORYSHIN, N. I. and AZARYAN, A. G. (1972) The role of circadian processes in insect photoperiodism. *Zhur. obshch. Biol.* **33**, 21-31. (In Russian.)
- UEDA, H.R., MATSUMOTO, A., KAWAMURA, M., IINO, M., TANIMURA, T. and HASHIMOTO, S. (2002) Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. *J. Biol. Chem.* **277**, 14048-14052.
- UMEYA, K. and MASAKI, S. (1969) Biology of *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) in Japan, VII. Delayed development of summer pupae. *Res. Bull. Pl. Prot. Japan*, **7**, 1-16.
- UNDERWOOD, H. (1977) Circadian organization in lizards: the role of the pineal organ. *Science, Wash.* **195**, 587-589.

- USUA, E. J. (1973) Induction of diapause in the maize stemborer, *Busseola fusca*. *Ent. exp.appl.* **16**, 322-328.
- VAFOPOULOU, X., SIM, C.-H. and STEEL, C.G.H. (1996) Prothoracicotrophic hormone in *Rhodnius prolixus*: *in vitro* analysis and changes in amounts in the brain and retrocerebral complex during larval-adult development. *J. Insect Physiol.* **42**, 407-415.
- VAFOPOULOU, X. and STEEL, C.G.H. (1989) Developmental and diurnal changes in ecdysteroid biosynthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera) *in vitro* during the last larval instar. *Gen. Comp. Endocrin.* **74**, 484-493.
- VAFOPOULOU, X. and STEEL, C.G.H. (1991) Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: evidence of a dual oscillator system. *Gen. Comp. Endocrinol.* **83**, 27-34.
- VAFOPOULOU, X. and STEEL, C.G.H. (1992) *In vitro* photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen. Comp. Endocrin.* **86**, 1-9.
- VAFOPOULOU, X. and STEEL, C.G.H. (1996a) The insect neuropeptide prothoracicotrophic hormone is released with a daily rhythm: Re-evaluation of its role in development. *Proc. Nat. Acad. Sci. USA* **93**, 3368-3372.
- VAFOPOULOU, X. and STEEL, C.G.H. (1996b) Circadian regulation of a daily rhythm of release of prothoracicotrophic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen. Comp. Endocrin.* **102**, 123-129.
- VAFOPOULOU, X. and STEEL, C.G.H. (1998) A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J. comp. Physiol. A* **182**, 343-349.
- VAFOPOULOU, X. and STEEL, C.G.H. (1999) Daily rhythm of responsiveness to prothoracicotrophic hormone in prothoracic glands of *Rhodnius prolixus*. *Archives of Insect Biochemistry and Physiology* **41**, 117-123.
- VAFOPOULOU, X. and STEEL, C.G.H. (2001) Induction of rhythmicity in prothoracicotrophic hormone and ecdysteroids in *Rhodnius prolixus*: roles of photic and neuropeptide Zeitgebers. *J. Insect Physiol.* **47**, 935-941.
- VAFOPOULOU, X. and STEEL, C.G.H. (2002) Prothoracicotrophic hormone of *Rhodnius prolixus*: Partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. Submitted.
- VAFOPOULOU, X., STEEL, C.G.H. and TERRY, K.L. (2001) Ecdysteroid receptor (EcR) cycles with tissue-specific phases in the insect *Rhodnius prolixus*: relationships with circadian regulation of blood ecdysteroid levels. In: Goos, H.J.Th., Rastogi, R.K., Vaudry, H. And Pierantoni, R. (Eds.). *Perspective in Comparative Endocrinology: Unity and Diversity*. Monduzzi Editory, Bologna, pp. 837-844.
- VAN GELDER, R.N., BAE, H., PALAZZOLO, M.J. and KRASNOW, M.A. (1995) Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillatory mRNAs in the fly head. *Current Biol.* **5**, 1424-1436.
- VAN GELDER, R.N. and KRASNOW, M.A. (1996) A novel circadianly expressed *Drosophila melanogaster* gene dependent on the *period* gene for its rhythmic expression. *EMBO J.* **15**, 1625-1631.
- VAN HOUTEN, Y.M., OVERMEER, W.P.J., VAN ZON, A.Q. and VEERMAN, A. (1988). Thermoperiodic induction of diapause in the predacious mite, *Amblyseius potentillae*. *J. Insect Physiol.* **34**, 285-290.
- VAN HOUTEN, Y.M., OVERMEER, W.P.J. and VEERMAN, A. (1987) Thermoperiodically induced diapause in a mite in constant darkness is vitamin A dependent. *Experientia* **43**, 933-935.
- VAN HOUTEN, Y.M. and VEENENDAAL, R.L. (1990) Effects of photoperiod, temperature, food and relative humidity on the induction of diapause in the predatory mite *Amblyseius potentillae*. *Exp. Appl. Acarol.* **10**, 111-128.
- VAN HOUTEN, Y.M. and VEERMAN, A. (1990) Photoperiodism and thermoperiodism in the predatory mite *Amblyseius potentillae* are probably based on the same mechanism. *J. comp. Physiol. A* **167**, 201-209.
- VAN ZON, A. Q., OVERMEER, W. P. J. and VEERMAN, A. (1981) Carotenoids are functionally involved in photoperiodic induction of diapause in a predacious mite. *Science, Wash.* **213**, 1131-1133.
- VAZ NUNES, M. (1981) A 'simple clock' approach to circadian rhythms: an easy way to predict the clock's singularity. *J. theoret. Biol.* **92**, 227-239.
- VAZ NUNES, M. (1990) The effect of temperature on photoperiodic induction of diapause in insects and mites: a model for the photoperiodic "counter". *J. theoret. Biol.* **146**, 369-378.
- VAZ NUNES, M. (1994) A pacemaker-slave model for the photoperiodic clock in the vetch aphid, *Megoura viciae*. *J. Insect Physiol.* **40**, 651-659.
- VAZ NUNES, M. (1998) A double circadian oscillator model for quantitative photoperiodic time measurement in insects and mites. *J. theoret. Biol.* **194**, 299-311.
- VAZ NUNES, M. and HARDIE, J. (1987) An instantly damping oscillator model for photoperiodic time measurement in the aphid *Aphis fabae*. *J. Insect Physiol.* **33**, 831-841.

- VAZ NUNES, M. and HARDIE, J. (1989) "Bistability" experiments and photoperiodic morph determination in the aphid *Aphis fabae*. *Physiol. Entomol.* **14**, 107-113.
- VAZ NUNES, M. and HARDIE, J. (1993) Circadian rhythmicity is involved in photoperiodic time measurement in the aphid *Megoura viciae*. *Experientia* **49**, 711-713.
- VAZ NUNES, M. and HARDIE, J. (1994) The photoperiodic counter in the black bean aphid, *Aphis fabae*. *J. Insect Physiol.* **40**, 827-834.
- VAZ NUNES, M. and HARDIE, J. (1999) The effect of temperature on the photoperiodic 'counters' for female morph and sex determination in two clones of the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* **24**, 339-345.
- VAZ NUNES, M., KENNY, N.A.P. and SAUNDERS, D.S. (1990) The photoperiodic clock in the blowfly *Calliphora vicina*. *J. Insect Physiol.* **36**, 61-67.
- VAZ NUNES, M., KOVEOS, D.S. and VEERMAN, A. (1990) Geographical variation in photoperiodic induction in the spider mite (*Tetranychus urticae*): A causal relation between critical nightlength and circadian period? *J. Biol. Rhythms* **5**, 47-57.
- VAZ NUNES, M., LEWIS, R.D. and SAUNDERS, D.S. (1991) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites. I. The basic control system as a model for circadian rhythms. *J. theoret. Biol.* **152**, 287-298.
- VAZ NUNES, M. and SAUNDERS, D.S. (1989) The effect of larval temperature and photoperiod on the incidence of larval diapause in the blowfly, *Calliphora vicina*. *Physiol. Entomol.* **14**, 471-474.
- VAZ NUNES, M. and SAUNDERS, D.S. (1999) Photoperiodic time measurement in insects: a review of clock models. *J. Biol. Rhythms* **14**, 84-104.
- VAZ NUNES, M., SAUNDERS, D.S. and LEWIS, R.D. (1991) A coupled oscillator feedback system as a model for the photoperiodic clock in insects and mites. II. Simulations of photoperiodic responses. *J. theoret. Biol.* **152**, 299-317.
- VAZ NUNES, M. and VEERMAN, A. (1979a) Photoperiodic time measurement in spider mites. I. Development of a two interval timers model. *J. comp. Physiol.* **134**, 203-217.
- VAZ NUNES, M. and VEERMAN, A. (1979b) Photoperiodic time measurement in spider mites. II. Effects of skeleton photoperiods. *J. comp. Physiol.* **134**, 219-226.
- VAZ NUNES, M. and VEERMAN, A. (1982) Photoperiodic time measurement in the spider mite *Tetranychus urticae*: A novel concept. *J. Insect Physiol.* **28**, 1041-1053.
- VAZ NUNES, M. and VEERMAN, A. (1984) Light-break experiments and photoperiodic time measurement in the spider mite, *Tetranychus ulmi*. *J. Insect Physiol.* **30**, 891-897.
- VAZ NUNES, M. and VEERMAN, A. (1986a) A "dusk" oscillator affects photoperiodic induction of diapause in the spider mite, *Tetranychus urticae*. *J. Insect Physiol.* **32**, 605-614.
- VAZ NUNES, M. and VEERMAN, A. (1986b) Similarities between the photoperiodic clocks of *Megoura* and *Tetranychus*: experiments with spider mites. *J. Insect Physiol.* **32**, 1029-1034.
- VAZ NUNES, M. and VEERMAN, A. (1997) "Bistability" experiments and the photoperiodic clock in the spider mite *Tetranychus urticae*. *Entomol. Exp. Appl.* **84**, 195-197.
- VAZ NUNES, M., KOVEOS, D.S. and VEERMAN, A. (1990) Geographical variation in photoperiodic induction of diapause in the spider mite (*Tetranychus urticae*): A causal relation between critical night length and circadian period? *J. Biol. Rhythms* **5**, 47-57.
- VEERMAN, A. (1977a) Aspects of the induction of diapause in a laboratory strain of the mite *Tetranychus urticae*. *J. Insect Physiol.* **23**, 703-711.
- VEERMAN, A. (1977b) Photoperiodic termination of diapause in spider mites. *Nature, Lond.* **266**, 526-527.
- VEERMAN, A. (1980) Functional involvement of carotenoids in photoperiodic induction of diapause in the spider mite, *Tetranychus urticae*. *Physiol. Ent.* **5**, 291-300.
- VEERMAN, A. (1998) Temperature effects on the photoperiodic response of the spider mite *Tetranychus urticae*. Vith European Congress of Entomology, Ceke Budejovice 23 – 29th August 1998.
- VEERMAN, A., BEEKMAN, M. and VEENENDAAL, R.L. (1988) Photoperiodic induction of diapause in the large white butterfly, *Pieris brassicae*: Evidence for hourglass time measurement. *J. Insect Physiol.* **34**, 1063-1069.
- VEERMAN, A. and HELLE, W., (1978) Evidence for the functional involvement of carotenoids in the photoperiodic reaction of spider mites. *Nature, Lond.* **275**, 234.
- VEERMAN, A. and HERREBOUT, W.M. (1982) Photoperiodic response curve for *Yponomeuta vigintipunctatus* (Retz.) (Lepidoptera: Yponomeutidae). *Netherlands J. Zool.* **32**, 117-122.
- VEERMAN, A. and KOVEOS, D.S. (1989) Separation of photoperiodic and circadian effects on the termination of diapause in the spider mite *Tetranychus urticae*. *Experientia* **45**, 1143-1146.

- VEERMAN, A., OVERMEER, W.P.J., VAN ZON, A.Q., DE BOER, J.M., DE WAARD, E.R. and HUISMAN, H.O. (1983) Vitamin A is essential for photoperiodic induction of diapause in an eyeless mite. *Nature* **302**, 248-249.
- VEERMAN, A., SLAGT, M.E., ALDERLIEST, M.F.J. and VEENENDAAL, R.L. (1985) Photoperiodic induction of diapause in an insect is vitamin A dependent. *Experientia* **41**, 1194-1195.
- VEERMAN, A. and VAZ NUNES, M. (1980) Circadian rhythmicity participates in the photoperiodic determination of diapause in spider mites. *Nature, Lond.* **287**, 140-141.
- VEERMAN, A. and VAZ NUNES, M. (1984) Photoperiod reception in spider mites: photoreceptor, clock and counter. In *Photoperiodic Regulation of Insect and Molluscan Hormones*. Ciba Foundation Symposium 104, pp 48-64.
- VEERMAN, A. and VAZ NUNES, M. (1987) Analysis of the operation of the photoperiodic counter provides evidence for hourglass time measurement in the spider mite *Tetranychus urticae*. *J. comp. Physiol. A* **160**, 421-430.
- VEPSÄLÄINEN, K. (1971a) The roles of photoperiodism and genetic switch in alary polymorphism in *Gerris* (Het., Gerridae) (a preliminary report). *Acta Ent. Fennica*, **29**, 101-102.
- VEPSÄLÄINEN, K. (1971b) The role of gradually changing daylength in determination of wing length, alary dimorphism and diapause in a *Gerris odontogaster* (Zett.) population (Gerridae, Heteroptera) in South Finland. *Ann.Acad. Sci. Fenn.A IV Biologica*, **183**, 1-25.
- VINOGRADOVA, E. B. (1960) The experimental investigation of ecological factors inducing imaginal diapause in bloodsucking mosquitoes (Diptera, Culicidae). *Ent. Obozr.* **39**, 327-340. (In Russian.)
- VINOGRADOVA, E. B. (1967) The effect of photoperiodism upon the larval development and the appearance of diapausing eggs in *Aedes triseriatus* Say (Diptera, Culicidae). *Parasitologia*, **1**, 19-26. (in Russian.)
- VINOGRADOVA, E.B. (1975) Intraspecific variability of reactions controlling the larval diapause in *Calliphora vicina* R.-D. (Diptera, Calliphoridae). *Rev. d'Entomol. de l'URSS* **4**, 720-735 [In Russian, English Summary].
- VINOGRADOVA, E. B. (1976) Embryonic photoperiodic sensitivity in two species of flesh flies, *Parasarcophaga similis* and *Boettcherisca septendrionalis*. *J. Insect Physiol.* **22**, 819-822.
- VINOGRADOVA, E. B. and ZINOVJEVA, K. B. (1972a) Experimental investigation of the seasonal aspect of the relationship between blowflies and their parasites. *J. Insect Physiol.* **18**, 1629-1638.
- VINOGRADOVA, E. B. and ZINOVJEVA, K. B. (1972b) Maternal induction of larval diapause in the blowfly, *Calliphora vicina*. *J. Insect Physiol.* **18**, 2401-2409.
- VIVIANI, V.R. and BECHARA, E.J. H. (1997) Bioluminescence and biological aspects of Brazilian railroad-worms (Coleoptera: Phengodidae). *Ann. Ent. Soc. Am.* **90**, 389-398.
- VIVIEN-ROELS, B., PEVET, P., BECK, O. and FEVRE-MONTANGE, M. (1984) Identification of melatonin in the compound eyes of an insect, the locust (*Locusta migratoria*), by radioimmunoassay and gas chromatography-mass spectrometry. *Neurosci. Letters* **49**, 153-157.
- VON SCHILCHER, F. and HALL, J.C. (1979) Neural topography of courtship song in sex mosaics of *Drosophila melanogaster*. *J. comp. Physiol. A* **129**, 85-95.
- VUILLAUME, M., SEUGE, J. and BERGERARD, J. (1974) Pigment, photoreception and nymph diapause of *Pieris brassicae*. *Int. J. Chronobiol.* **2**, 181-188.
- WADDELL, B., LEWIS, R.D. and ENGELMANN, W. (1990) Localization of the circadian pacemakers of *Hemideina thoracica* (Orthoptera: Stenopelmatidae). *J. Biol. Rhythms* **5**, 131-139.
- WAHL, O. (1932) Neue Untersuchungen über das Zeitgedächtnis der Bienen. *Z. vergl. Physiol.* **16**, 529-589.
- WALKER, G. P. and DENLINGER, D. L. (1980) Juvenile hormone and moulting hormone titres diapause and non-diapause destined flesh flies. *J. Insect Physiol.* **26**, 661-664.
- WALKER, W. F. (1978) Mating behaviour in *Oncopeltus fasciatus* (Dallas): effects of diet, photoperiod, juvenoids and precocene II. *Physiol. Ent.* **3**, 147-155.
- WALKER, W.F. (1979) Mating behaviour in *Oncopeltus fasciatus*: circadian rhythms of coupling, copulation duration and 'rocking' behaviour. *Physiol. Entomol.* **4**, 275-283.
- WALKER, W.F. (1979) Mating behaviour in *Oncopeltus fasciatus*: circadian rhythms of coupling, copulation duration and 'rocking' behaviour. *Physiol. Entomol.* **4**, 275-283.
- WARDHAUGH, K. G. (1977) The effects of temperature and photoperiod on the morphology of the egg pod of the Australian plague locust (*Chortoicetes terminifera* Walker, Orthoptera: Acrididae). *Aust. J. Ecol.* **2**, 81-88.
- WARMAN, G.R. (1995) Circadian biology of the Australian sheep blowfly *Lucilia cuprina*. *M.Sc. thesis University of Auckland, New Zealand*.

- WARMAN, G.R. and LEWIS, R.D. (2001) Molecular simulation modelling of the circadian system of the blow fly, *Lucilia cuprina*. *J. Insect Physiol.* **47**, 923-934.
- WARMAN, G.R., NEWCOMB, R.D., LEWIS, R.D., and EVANS, C.W. (2000) Analysis of the circadian clock gene *period* in the sheep blow fly *Lucilia cuprina*. *Genet. Res.* **75**, 257-267.
- WASSMER, G.T., CAIN, W. and PAGE, T.L. (1996) Photoperiodic regulation of haemolymph protein in the woodroach, *Parcoblatta pensylvanicus*. *J. Insect Physiol.* **42**, 851-858.
- WASSMER, G.T. and PAGE, T.L. (1993) Photoperiodic time measurement with a graded response in a cockroach. *J. Biol. Rhythms* **8**, 47-56.
- WATT, W. B. (1968) Adaptive significance of pigment polymorphisms in *Colias* butterflies. I. Variation of melanin pigment in relation to thermoregulation. *Evolution*, **22**, 437-458.
- WATT, W. B. (1969) Adaptive significance of pigment polymorphisms in *Colias* butterflies. II. Thermoregulation and photoperiodically controlled melanism variation in *Colias eurytheme*. *Proc. Nat. Acad. Sci. U.S.A.* **63**, 767-774.
- WAY, M. J. and HOPKINS, B. A. (1950) The influence of photoperiod and temperature on the induction of diapause in *Diatraea oleracea* L. *J. exp. Biol.* **27**, 365-376.
- WEBER, F. (1965) Zur Tagaktivität von *Carabus*-Arten. *Zool. Anz.* **175**, 354-360.
- WEBER, F. (1967) Die Periodenlänge der circadianen Laufperiodizität bei drei *Carabus*-Arten (Coleoptera, Ins.). *Naturwiss.* **54**, 122.
- WEBER, F. (1995) Cyclic layer deposition in the cockroach (*Blaberus craniifer*) endocuticle: a circadian rhythm in leg pieces cultured *in vitro*. *J. Insect Physiol.* **41**, 153-161.
- WEBSTER, R.P. and CARDE, R.T. (1982) Relationships among pheromone titre, calling and age in the omnivorous leaf-roller moth (*Platynota stultana*). *J. Insect Physiol.* **28**, 925-934.
- WEBSTER, R.P. and YIN, C.M. (1997) Effects of photoperiod and temperature on calling behaviour of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). *Can. Ent.* **129**, 843-854.
- WELLSO, S. G. and ADKISSON, P. L. (1966) A long-day short-day effect in the photoperiodic control of the pupal diapause of the bollworm, *Heliothis zea* (Bodie) (Lepidoptera: Noctuidae). *J. Insect Physiol.* **12**, 1455-1465.
- WENT, F. W. (1959) The periodic aspect of photoperiodism and thermoperiodicity. In *Photoperiodism and Related Phenomena in Plants and Animals* (Ed. WITHROW, R. B.), pp. 551-564. Am. Ass. Adv. Sci., Washington.
- WERNER, G. (1954) Tanze und Zeitempfinden der Honigbiene in Abhängigkeit vom Stoffwechsel. *Z. vergl. Physiol.* **36**, 464-487.
- WESTBROOK, A.L. and BOLLENBACHER, W.E. (1990) The development of identified neurosecretory cells in the tobacco hornworm, *Manduca sexta*. *Develop. Biology* **140**, 291-299.
- WETTERBERG, L., HAYES, D.K. and HALBERG, F. (1987) Circadian rhythms of melatonin in the brain of the face fly, *Musca autumnalis* De Geer. *Chronobiologia* **14**, 377-381.
- WEVER, R. (1965) A mathematical model for circadian rhythms. In *Circadian Clocks* (Ed. ASCHOFF, J.), pp. 47-63. North-Holland, Amsterdam.
- WEVER, R. (1972) Virtual synchronization towards the limits of the range of entrainment. *J. theoret. Biol.* **36**, 119-132.
- WEVER, R. (1975) The circadian multioscillator system of man. *Int. J. Chronobiol.* **3**, 19-56.
- WHEELER, D.A., HAMBLIN-COYLE, M.J., DUSHAY, M.S. and HALL, J.C. (1993) Behavior in light-dark cycles of *Drosophila* mutants that are arrhythmic, blind or both. *J. Biol. Rhythms* **8**, 67-94.
- WHITE, L., RINGO, J. and DOWSE, H. (1992) The effects of deuterium oxide and temperature on heart rate in *Drosophila*. *J. Comp. Physiol. B* **162**, 278-283.
- WHITESSELL, J. J. and WALKER, T. J. (1978) Photoperiodically determined dimorphic calling songs in a katydid. *Nature, Lond.* **274**, 887-888.
- WIEDENMANN, G. (1977a) Two activity peaks in the circadian rhythm of the cockroach *Leucophaea maderae*. *J. interdiscipl. Cycle Res.* **8**, 378-383.
- WIEDENMANN, G. (1977b) No 'point of singularity' in the circadian activity rhythm of the cockroach *Leucophaea maderae*. *Chronobiol.* **4**, 165.
- WIEDENMANN, G. (1977c) Weak and strong phase shifting in the activity rhythm of *Leucophaea maderae* (Blaberidae) after light pulses of high intensity. *Z. Naturforsch.* **32C**, 464-465.
- WIEDENMANN, G. (1980b) Two peaks in the activity rhythm of cockroaches controlled by one circadian pacemaker. *J. comp. Physiol.* **137**, 249-254.
- WIEDENMANN, G. (1983) Splitting in a circadian activity rhythm: the expression of bilaterally paired oscillators. *J. Comp. Physiol. A* **150**, 51-60.

- WIEDENMANN, G. and LOHER, W. (1984) Circadian control of singing in crickets: two different pacemakers for early evening and before dawn activity. *J. Insect Physiol.* **30**, 145-151.
- WIEDENMANN, G., LUKAT, R. and WEBER, F. (1986) Cyclic layer deposition in the cockroach endocuticle: A circadian rhythm? *J. Insect Physiol.* **32**, 1019-1027.
- WIEDENMANN, G., KRÜGER-ALEF, K. and MARTIN, W. (1988) The circadian control of calling song and walking activity patterns in male crickets (*teleogryllus commodus*). *Exp. Biol.* **47**, 127-137.
- WIGGLESWORTH, V.B. (1952) The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. Exp. Biol.* **29**, 561-570.
- WIGGLESWORTH, V.B. (1985) Historical Perspective. In: Kerkut, G.A. and Gilbert, L.I. (Eds.). *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 7, Pergamon Press, Oxford, pp. 1-24.
- WILDE, J. DE (1958) Perception of the photoperiod by the Colorado potato beetle (*Leptinotarsa decemlineata* Say.). *Proc. Xth Int. Congr. Ent. Montreal 1956*, **2**, 213-218.
- WILDE, J. DE (1962) Photoperiodism in insects and mites. *A. Rev. Ent.* **7**, 1-26.
- WILDE, J. DE and BOER, J. A. DE (1961) Physiology of diapause in the adult Colorado beetle - II. Diapause as a case of pseudoallatectomy. *J. Insect Physiol.* **6**, 152-161.
- WILDE, J. DE and BONGA, H. (1958) Observations on threshold intensity and sensitivity of of different wave lengths of photoperiodic response in the Colorado beetle (*Leptinotarsa decemlineata* Say). *Entomologia exp. appl.* **1**, 301-307.
- WILDE, J. DE, DUINTJER, C. S. and MOOK, L. (1959) Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata*). I. The photoperiod as a controlling factor. *J. Insect Physiol.* **3**, 75-85.
- WILLIAMS, C. M. (1946) Physiology of insect diapause. I. The role of the brain in the production and termination of pupal dormancy in the giant silkworm *Platysamia cecropia*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **90**, 234-243.
- WILLIAMS, C. M. (1952) Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **103**, 120-138.
- WILLIAMS, C. M. (1963) Control of pupal diapause by the direct action of light on the insect brain. *Science, Wash.* **140**, 386.
- WILLIAMS, C. M. (1967) The present status of the brain hormone. In *Insects and Physiology* (Ed. BEAMENT, J. W. L. and TREHERNE, J. E.), pp. 133-139. Oliver & Boyd, Edinburgh and London.
- WILLIAMS, C. M. (1969a) Photoperiodism and the endocrine aspects of insect diapause. *Symp. Soc. exp. Biol.* **23**, 285-300.
- WILLIAMS, C. M. (1969b) Nervous and hormonal communication in insect development. *Dev. Biol. Supp.* **3**, 133-150.
- WILLIAMS, C. M. and ADKISSON, P. L. (1964) Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, *Antheraea pernyi*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **127**, 511-525.
- WILLIAMS, C. M., ADKISSON, P. L. and WALCOTT, C. (1965) Physiology of insect diapauses XV. The transmission of photoperiodic signals to the brain of the oak silkworm, *Antheraea pernyi*. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **128**, 497-507.
- WILLIAMS, K.D. and SOKOLOVSKI, M.B. (1993) Diapause in *Drosophila melanogaster* females: a genetic analysis. *Heredity* **71**, 312-317.
- WILLS, S.A., PAGE, T.L. and COLWELL, C.S. (1985) Circadian rhythms in the electroretinogram of the cockroach. *J. Biol. Rhythms* **1**, 25-37.
- WILSON, T. and HASTINGS, J.W. (1998) Bioluminescence. *Ann. Rev. Cell Develop. Biol.* **14**, 197-230.
- WINFREE, A.T. (1967) Biological rhythms and the behavior of populations of coupled oscillators. *J. theor. Biol.* **16**, 15-42.
- WINFREE, A. T. (1970a) The temporal morphology of a biological clock. In *Lectures on Mathematics in the Life Sciences* (Ed. GERSTENHABER, M.), pp. 111-150. American Mathematical Society, Providence, R.I.
- WINFREE, A. T. (1970b) Integrated view of resetting a circadian clock. *J. theore. Biol.* **28**, 327-374.
- WINFREE, A.T. (1971) Corkscrews and singularities in fruitflies: resetting behavior of the circadian eclosion rhythm. In *Biochronometry* (Ed. MENAKER, M.), pp. 81-109. National Academy of Sciences, Washington.
- WINFREE, A. T. (1972a) Slow dark adaptation in *Drosophila's* circadian clock. *J. comp. Physiol.* **77**, 418-434.
- WINFREE, A. T. (1972b) Acute temperature sensitivity of the circadian rhythm in *Drosophila*. *J. Insect Physiol.* **18**, 181-185.
- WINFREE, A. T. (1973a) Time and timelessness in biological clocks. In: *Temporal Aspects of Therapeutics* (Ed. URQUARDT, J. and YATES, F. E.), pp. 35-57. Plenum Press, New York.
- WINFREE, A. T. (1973b) Resetting the amplitude of *Drosophila's* circadian chronometer. *J. comp. Physiol.* **85**, 105-140.

- WINFREE, A. T. (1974) Suppressing *Drosophila*'s circadian rhythm with dim light. *Science, Wash.* **183**, 970-972.
- WINFREE, A. T. (1980) *The Geometry of Biological Time*. Springer-Verlag, New York, Heidelberg, Berlin.
- WINFREE, A. T. and GORDON, H. (1977) The photosensitivity of a mutant circadian clock. *J. comp. Physiol.* **122**, 87-109.
- WOJTASEK, H. and PRESTWICK, G.D. (1995) Key disulfide bonds in an insect hormone binding protein: cDNA cloning of a juvenile hormone binding protein of *Heliothis virescens* and ligand binding by native and mutant forms. *Biochemistry* **34**, 5234-5241.
- WOLDA, H. and DENLINGER, D.L. (1984) Diapause in a large aggregation of a tropical beetle. *Ecol. Entomol.* **9**, 217-230.
- WOLF, E. (1927) Über das Heimkehrvermögen der Beinen. *Z. vergl. Physiol.* **6**, 221-254.
- WUNDERER, H. and KRAMER, J.J. de (1989) Dorsal ocelli and light induced diurnal activity patterns in the Arctiid moth *Cretonotus transiens*. *J. Insect Physiol.* **35**, 87-95.
- WYATT, G.R. and DAVEY, K.G. (1996) Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. In: Evans, P.D. (Ed), *Advances in Insect Physiology*, Academic Press, London, vol 26, pp. 1-155.
- YAGI, S. and FUKAYA, M. (1974) Juvenile hormone as a key factor regulating larval diapause of the rice stem borer *Chilo suppressalis* (Lepidoptera: Pyralidae). *Appl. ent. Zool.* **9**, 247-255.
- YAGITA, K., TAMANINI, F., van der HORST, C.T.J. and OKAMURA, H. (2001) Molecular mechanisms of the biological clock in cultures fibroblasts. *Science* **292**, 278-281.
- YAMAOKA, K. and HIRAO, T. (1977) Stimulation of virginal oviposition by male factor and its effect on spontaneous nervous activity in *Bombyx mori*. *J. Insect Physiol.* **23**, 57-63.
- YAMAOKA, K. and HIRAO, T. (1981) Mechanisms of ovipositional behaviour in *Bombyx mori*: Time-gating and accumulation of the internal factor. *Intern. J. Invertebrate Reproduction* **4**, 169-180.
- YASUYAMA, K. and MEINERTZHAGEN, I.A. (1999) Extraretinal photoreceptors at the compound eye's posterior margin in *Drosophila melanogaster*. *J. Comp. Neurol.* **412**, 193-202.
- YIN, C.-M. and CHIPPENDALE, G. M. (1973) Juvenile hormone regulation of the larval diapause of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect. Physiol.* **19**, 2403-2420.
- YOSHIDA, T. and KIMURA, M.T. (1993) The photoperiodic clock of *Drosophila triauraria*: involvement of a circadian oscillatory system. *J. Insect Physiol.* **39**, 223-228.
- YOUTHED, G. J. and MORAN, V. C. (1969a) The solar-day activity rhythm of Myrmeleontid larvae. *J. Insect Physiol.* **15**, 1103-1116.
- YOUTHED, G. J. and MORAN, V. C. (1969b) The lunar-day activity rhythm of Myrmeleontid larvae. *J. Insect Physiol.* **15**, 1259-1271.
- ZABIROV, S. M. (1961) Factors governing the seasonal development cycles of the spinach leaf miner (*Pegomya hyoscyami* Panz.) and the cabbage maggot (*Hylemyia brassicae* Bouche) (Diptera, Anthomyiidae). *Ent. Obozr.* **40**, 148-151. (In Russian.)
- ZASLAVSKI, V.A. (1988) *Insect Development, Photoperiodic and Temperature Control*. Springer-Verlag.
- ZASLAVSKII, V.A. (1996) Essentials of the environmental control of insect seasonality as reference points for comparative studies in other invertebrates. *Hydrobiologia* **320**, 123-130.
- ZASLAVSKI, V.A. and FOMENKO, R.B. (1983) Quantitative photoperiod perception in the aphid *Megoura viciae* Buckt. (Homoptera, Aphididae). *Entomol. Rev.* **62**, 1-10.
- ZDAREK, J. and DENLINGER, D. L. (1975) Action of ecdysoids, juvenoids, and new hormonal agents on the termination of pupal diapause in the flesh fly. *J. Insect Physiol.* **21**, 1193-1202.
- ZDAREK, J., DENLINGER, D.L. and OTIENO, L.H. (1992) Does the tsetse parturition rhythm have a circadian basis? *Physiol. Entomol.* **17**, 305-307.
- ZDAREK, J. and DENLINGER, D.L. (1995) Changes in temperature, not photoperiod, control the pattern of adult eclosion in the tsetse, *Glossina morsitans*. *Physiol. Entomol.* **20**, 362-366.
- ZEEUW, D. DE (1957) Flowering of *Xanthium* under long-day conditions. *Nature, Lond.* **180**, 558.
- ZELAZNY, B. and NEVILLE, A.C. (1972) Endocuticle layer formation controlled by non-circadian clocks in beetle. *J. Insect Physiol.* **18**, 1967-1979.
- ZENG, H., QIAN, Z., MYERS, M.P. and ROSBASH, M. (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature* **380**, 129-135.
- ZHUKOVSKAYA, M.I. (1995) Circadian rhythm of sex pheromone perception in the male American cockroach, *Periplaneta americana* L. *J. Insect Physiol.* **41**, 941-946.

- ZIMMERMAN, W. F. (1969) On the absence of circadian rhythmicity in *Drosophila pseudoobscura* pupae. *Biol. Bull. Mar. Biol. Lab., Woods Hole*, **136**, 494-500.
- ZIMMERMAN, W. F. and GOLDSMITH, T. H. (1971) Photosensitivity of the circadian rhythm and of visual receptors in carotenoid depleted *Drosophila*. *Science, Wash.* **171**, 1167-1168.
- ZIMMERMAN, W. F. and IVES, D. (1971) Some photophysiological aspects of circadian rhythmicity in *Drosophila*. In *Biochronometry* (Ed. MENAKER, M.), pp. 381-391. National Academy of Sciences, Washington.
- ZIMMERMAN, W. F., PITTENDRIGH, C. S. and PAVLIDIS, T. (1968) Temperature compensation of the circadian oscillation in *Drosophila pseudoobscura* and its entrainment by temperature cycles. *J. Insect Physiol.* **14**, 669-684.
- ZINOVYEVA, K. B. and POLYAKOVA, D. I. (1987) Effect of photoperiod and thermoperiod on the daily eclosion rhythm in selected lines of *Calliphora vicina* R.-D. (Diptera, Calliphoridae). *Ent. Obozr.* **2**, 236-246 [In Russian].
- ZITNAN, D. and ADAMS, M. E. (2000) Excitatory and inhibitory roles of central ganglia in initiation of the insect ecdysis behavioural sequence. *J. exp. Biol.* **203**, 1329-1340.
- ZITNAN, D., KINGAN, T. G. and BECKAGE, N. E. (1995) Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. Mol. Biology* **25**, 669-678.
- ZITNAN, D., KINGAN, T. G., HERMESMAN, J. L. and ADAMS, M. E. (1996) Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science* **271**, 88-91.
- ZITNAN, D., ROSS, L. S., ZITNANOVA, I., HERMESMAN, J. L., GILL, S. S. and ADAMS, M. E. (1999) Steroid orchestration of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron* **23**, 523-535.

INDEX

- Abnormal light/dark cycles 350
Abraxas miranda 302, 314
 Accessory medulla 252, 260, 261
Acheta domesticus 9, 23, 34, 35, 58, 123, 160, 253, 255
 Acoustic communication 182
Acrolepia assectella 293
Acronycta rumicis
 effect of latitude 322, 388
 effect of longitude 326
 effect of temperature 307, 316, 386
 genetics 329, 330
 intensity thresholds 334
 light and dark periods 350
 photoperiodic counter 378, 379, 386
 photoperiodic response curve 300
 pupal diapause 275
 shortening day length 306
 spectral sensitivity 332, 333
 symmetrical skeleton photoperiods 361
Acrydium arenosum 272
 Action spectra 95
 daily growth layers 150
 phase shifts 36, 95
 photoperiodic responses 442
Acyrthosiphum pisum 291, 303, 352
Adoxophyes spp. 169, 332, 342, 346, 352
 Adult diapause 283
Aedes aegypti
 bimodal activity 9, 24, 192
 constant light 15
 entrainment 34
 gating 61
 oviposition rhythm 44, 51
 ovulation 164
 pupation 44, 61
 violation of Aschoff's rule 15
Aedes atropalpus 346, 348, 388, 391
Aedes sierrensis 323
Aedes taeniorrhynchus 9, 24, 29, 57, 206, 212
Aedes togoi 275
Aedes triseriatus 292, 319, 391
Aedes vittatus 60
Aelia acuminata 328
 Aestivation 273
 'After-effects' 196, 230, 234
Agrotis ipsilon 159, 160, 294
Agrotis occulta 292
Agrotis triangulum 292
 Alary dimorphism 289
Aleurochiton complanatus 290, 318
Aleyrodes proletella 353
Alysia manducator 320
Amblyseius potentillae 309, 353, 442
Ameronothrus marinus 465
 Amplitude model 397, 402
Anacrydium aegyptium 283, 284, 434, 437
Anagasta kuhniella 44, 49, 88
Anaphothrips obscurus 290
Anastrepha suspensa 168
Anax imperator 306
Andevidia peponis 172
 Annual rhythms 466, 467, 468, 469
Anopheles farauti 24
Anopheles gambiae 9, 23, 34, 35, 58
 Antennal rhythms 118, 177
Antheraea pernyi
 ecdysis 140
 eclosion rhythm 263, 264
 effect of temperature 314
 flight activity 259
 gating 60
 genetics 328
 intensity thresholds 334, 335
 larval ecdyses 266
 light and dark periods 350
 location of clock 438
 PER/TIM cells 128, 153, 154, 266, 476
 photoperiodic clock 328, 446
 photoperiodic counter 378
 photoreceptors 263, 434, 438
 pupal diapause 275, 279
 pupal eclosion 4, 49, 60
 sensitive stage 310, 311
 spectral sensitivity 332
Antheraea yamamai 285
Anthonomus grandis 332
Anthrenus verbasci 60, 306, 466, 467, 468, 469
 Antifreeze protein 286
Anurida maritima 465
 A-oscillator (or pacemaker) 66, 68
Apanteles glomeratus 320, 443
Apanteles spuria 320
Apharaeta minuta 320
Aphis fabae 291, 346, 353, 370, 373, 387, 389
Aphis forbesi 272, 291
Apis mellifera
 JH titres 158
 neurosecretory cells 124
 non-photic Zeitgeber 39
 sleep-like state 10
 time-compensated sun orientation 454
 Zeitgedächtnis 449
 Apolysis 140
Aporia crataegi 320
Araschnia levana 287, 289, 311

- Arctic, rhythms in 13, 14
- Arrhythmicity 23, 47, 196
- Aschoff's rule 14, 223
- Ascia monuste* 287
- Asymmetrical skeletons 341, 422, 424
- Atrachya menetriesi* 328
- Barbara colfaxiana* 285
- Bdella interrupta* 465
- Bellieria melanura* 320
- Bilateral symmetry of pacemakers 198
- Bimodality 21, 22, 23, 191
- Biological clocks 3
- Bioluminescence 178
- Bistability phenomenon 78, 361, 420, 421
- Blaberus craniifer* 151, 190, 248
- Blaberus fuscus* 204
- Blaps gigas* 199, 256
- Blattella germanica* 35, 158
- Bombyx mori*
- ecdysis 140
 - embryonic diapause 274, 284
 - gut purge 268
 - intensity threshold 334
 - in vitro* PPTM 440
 - larval moulting 57
 - larval stemmata 434
 - maternal induction 311
 - phase response curve 64
 - pheromones 169
 - pupal eclosion 49
 - pupal moulting 124
 - pupation 58
 - PTTH 128, 268
 - short day response 272, 274, 302
 - spectral sensitivity 332
 - supplementary illumination 437
 - temperature effects 314
 - vitamin A 443
- Bombyxin 120
- B-oscillator (or slave) 66, 68
- Brain
- bisection 250
 - direct photoreception 257, 259, 264
 - location of clock 264
 - removal of 264
- Brain hormone (see PTTH)
- Brevicoryne brassicae* 291
- Bünning's hypothesis 340, 396, 428, 480
- Bünsow protocol 344
- Busseola fusca* 280
- Byrsotria fumigata* 9, 10, 16
- Calliphora erythrocephala* (see *vicina*)
- Calliphora stygia* 50
- Calliphora vicina*
- after effects 197
 - bistability experiment 362
 - brain photoreception 257
 - cold tolerance 286
 - damping oscillations 374
 - density effects 321
 - ecdysteroid agonist 475
 - entrainment 29, 34
 - FOS-like protein 258
 - free-running rhythm 10, 426
 - larval diapause 275, 281
 - lateral neurons 262
 - latitudinal cline 324, 325
 - maternal induction 281, 312
 - Nanda-Hamner exp 353, 356
 - optic lobe removal 257, 258, 434, 435, 441
 - phase response curve 38
 - photoperiodic counter 382, 386
 - pupae as hosts 390
 - range of entrainment 34
 - 'rebound effect' 27
 - selection for eclosion 99
 - selection for diapause 328, 329, 331
 - 'splitting' 193, 194
 - τ increase in LL 16, 17
 - temperature effects on counter 386, 387
 - temperature steps 38
- Camnula pellucida* 211
- Capnia bifrons* 306
- Carabus* spp. 14, 15, 29, 123, 256
- Carausius morosus* 254, 255
- Carotenoids 95, 108, 442
- Carpocapsa pomonella* 332, 427
- Cave-dwelling insects 13
- Cellular phenomena 1
- Central clocks 119
- Cephus cinctus* 277
- Ceratitis capitata* 55
- Ceutophilus conicaudus* 13
- Chaerodes trachyscelides* 465
- Changing day length 304, 305, 306
- Chaoborus americanus* 310, 318, 332, 378
- Chemical communication 168
- Chill tolerance 285
- Chilling 415
- Chilo suppressalis* 280
- Chironomus tentans* 138, 310
- Chloridea obsoleta* 318
- Cholera toxin 282
- Choristoneura fumiferana* 169, 328
- Chorthippus curtipennis* 253
- Chortoicetes terminifera* 297
- Chrysopa carnea* 297, 306, 323, 325
- Chrysopa downesi* 306
- Chrysopa mohave* 319
- Chymomyza costata* 50, 100, 321, 426

- Circa rhythms 2
 Circa-bi-dian rhythms 25
 Circadian rhythms
 developmental plasticity 25
 free-running period 10
 genetics 100, 103
 in individuals 7
 in orientation 453
 in photoperiodism 339, 341
 in populations 43
 in *Zeitgedächtnis* 449
 Circadian rule 14, 16
 Circadian system 189
 Circadian topography 351, 355, 357
 Circannual rhythms 449, 466, 481
 Clock 104
 Clock-commander model 397, 403
 Clock-Counter mechanism 395
 Clocks
 cellular 473
 complexity 41, 473, 476
 man-made, analogy 2
 site of 245, 444
Clunio balticus 460
Clunio marinus 100, 449, 455, 457, 482
Clunio mediterraneus 460
Clunio takahashii 460
Clunio tsushimensis 461
Coccinella septempunctata 295
 Coccinellid beetles 275, 294
Coeloides brunneri 312
Coelopa frigida 44
Cohiesta ignefusalis 280
 Cold tolerance 285
 Cold torpor 87
Colias eurytheme 287
 Coloration 287
 Complexity 473
Compoletus perdistinctus 297
 Compound eyes 107, 247, 253, 256, 441
Congenes punctiferalis 171
 Constant light, effects of 14, 80
 Control systems models 221
Corpora allata 283
Corpora cardiaca 122
Cothonaspis bouldardi 276, 309
 Courtship song rhythm 182, 184
Crambus tutillus 292, 306
Creotomus transiens 259
 Critical photoperiod 300, 301, 339
 Crustacean cardioactive peptide (CCAP) 146, 265
cryptochrome 105, 108, 480
Culex pipiens 24, 37, 83, 194, 235, 257, 296
Culex tarsalis 297
Culiseta spp 25, 235, 297
 Cuticle deposition 147
 cycle 104
 Cyclic nucleotides 282
Cydia (=Laspeyresia) pomonella 165, 167
 D₂O effects 20
Dacus dorsalis 50, 55
Dacus oleae 55
Dacus tryoni 27, 44, 49, 88
 Daily growth layers 147
 Damping rhythms 100, 371, 400
 Damping action of light 80
Danaus plexippus 294, 295
 Dark adaptation 87
 Day skipping 236, 237
 Delayed photoperiodic responses 311
Delia antiqua 328
Delphacodes striatella 290
Dendroides canadensis 286, 297, 353, 360
Dendrolimus pini 275, 332, 435
 Density effects 321
 Deposition of fat 297
 Developmental plasticity 26
 Diapause 3, 272, 273, 463
 Diapause hormone 284
 Diapause intensity 274
 Diapause reactivation 162, 279
 Diapause, summer 275
 Diapause 'syndrome' 274, 296
 Diapause, tropical 275
Dianemobius spp. 59, 254, 294, 324, 346, 352
Diataraxia oleracea 310, 314, 319, 334
Diatraea grandiosella 49, 53, 58, 80, 280, 297, 308, 324, 325, 443
Diatraea lineolata 280
Dicheirotichus gustavi 465
 Diet, effects of 318
Diploptera punctata 164
disconnected 258, 262
 Dormancy 273
 Double circadian oscillator model 397, 403
doubletime 104, 106
Drepanosiphum platanoides 292, 321
Drosophila, adult diapause 283
Drosophila alpina 283
Drosophila auraria 98, 283, 286, 324, 353
Drosophila deflexa 283
Drosophila littoralis 50, 96, 100, 283, 296, 324, 325, 328, 426
Drosophila melanogaster 103
 adult activity 21, 22
 arrhythmicity 196
 bimodality 194, 480
 carotenoid-free diet 95
 cell division 191
 courtship song 471
 developmental events 58, 59
 electric fields 39

- genes, tissue distr. 109, 190
 genetics of *period* 103
 microarrays 480
 mutants (see separate entries) 103, 258, 477
 Nanda-Hamner protocol 352
 nuclear sizes 124
 oviposition rhythm 52, 53
 phase response curve 33, 64
 photoperiodic response 283, 426, 427, 479
 pupal eclosion rhythm 50, 99
 recovery from heat stress 296
 reproductive diapause 283
 singularity 86
 survival 208, 209
 temperature compensation 477, 478
 temperature cycles 37
 ultradian rhythms 195, 471
Drosophila phalerata 283, 342
Drosophila pseudoobscura
 adult activity 21
 anoxia 87
 arrhythmicity 47
 bimodality 21, 23
 constant light 80
 damping action of light 80
 D₂O effects 47
 'early' and 'late' eclosion 99
 entrainment 63, 70, 71
 fixed point 71
 gating 58, 59, 60, 61
 'larval' and 'adult' clocks 206
 phase shifts 63
 phase-response curve 30, 32, 63, 64, 65, 67, 68, 69, 82
 pupal eclosion rhythm 2, 4, 44, 45, 202, 203
 range of entrainment 73
 singularity 84, 85, 86, 87, 209
 skeleton photoperiods 74, 75, 76, 77, 79
 spectral sensitivity 94, 95
 temperature pulses 48, 88, 89, 90, 91
 transfer to DD 81
 two-oscillator model 65, 66, 69
Drosophila rhythmically expressed genes (Dregs) 204
Drosophila simulans 471
Drosophila subobscura 50, 98
Drosophila triauraria 346, 353, 363
Drosophila victoria 44, 58
Drosophila virilis 210
 dsRNAi technology 481
 Dual role of light in photoperiodism 344
 Dual system theory (DST) 402
Dysaphis plantaginea 291
Dytiscus marginalis 283
 E-box 106
 Ecdysis 138, 145
 Ecdysteroids 40, 130, 143, 278, 279, 281, 289
 Ecdysteroid receptors 136, 138
 Eclosion hormone 141, 142, 265
 Ecdysis triggering hormone 146, 265
 'Egg' diapause 284
 Egg hatching rhythm 53, 152
 Electric fields 39
 Electoretinograms 199
 Embryonic diapause 284
Enallagma spp. 297
 Endocrine rhythms 123, 124, 130
 Endocrinology of diapause 276
 Endogenous rhythms 1, 8, 10
 Entrainment
 by light 28, 62, 70, 72, 112, 215, 225
 by temperature 35
 in photoperiodism 395, 418
Ephestia spp. 164, 321
Ephippiger sp. 254
Eriosechia brassicae 312, 319
Eucallipterus tiliac 292
 Eudiapause 274, 311
Euproctes similis 302
Eurema hecabe mandarina 287
Eurygaster integriceps 294
Euscelis lineolatus 290
Euscelis plebejus 290, 332
Euschistus spp. 290
 Exogenous light effects 23
 External coincidence model 397, 399, 405
 in *Pectinophora gossypiella* 343
 in *Sarcophaga argyrostoma* 400, 401
 Extra-retinal photoreceptors 253, 434
 Eye rhythms 199
 Feedback model 103, 213, 372, 373
 Fixed point 71
 Flight activity 8, 9
Formica rufa 455
Fos 258
 Free-running period 10, 229
 Freeze tolerance 285
 Frequency demultiplication 29
Galeruca tanaceti 283
Galleria mellonella 132
 Gamete formation and transport 161
 Gating 58
 Genetics of circadian rhythms 96, 99, 103
 Genetics of photoperiodism 327, 328, 329
 Genomics 107

- Geographical effects on photoperiodism 321
- Geotrupes sylvaticus* 14, 455
- Gerris odontogaster* 289, 294, 306
- glass 108
- Glossina morsitans* 9, 25, 51, 57, 94
- Gonotrophic dissociation 283
- Grapholitha molesta* 310, 314, 315, 332, 334, 341, 350, 378
- Graphosoma lineatum* 259
- Growth rates 292
- Gryllus bimaculatus* 33, 59, 80, 155, 204, 253, 254
- Gryllus campestris* 275
- Gut purge 124, 131
- Hadenocercus subterraneus* 13
- 'Hands' of the clock 414, 418, 425
- Head critical period 123, 124, 125
- Heat stress, recovery from 296
- Helicoverpa* spp. 44, 160, 169, 170, 171, 342
- Heliothis virescens* 278
- Hemideina thoracica*
- after-effects 196, 197
 - Aschoff's rule 15, 16
 - Clock models 213, 222, 223
 - compound eyes 253
 - day-skipping 237
 - oscillator location 255
 - phase response curve 226
 - scalloping 237
 - shattering 231
 - singularity? 32, 194
 - splitting 194, 229
 - spontaneous changes in τ 12, 230
 - temperature compensation 18, 19
 - temperature steps 227
- Hibernation 273
- Hofbauer-Büchner eyelet 107
- Holomelina* spp. 171
- Homeostasis of τ 17-20, 46, 47
- Homorocoryphus jezoensis* 88
- Hormonal regulation 122
- Host deprivation 319
- Host species, effect of 319, 320
- Hourglass-like clocks
- in *Clunio marinus* 460
 - in egg hatch rhythm 55
 - as heavily damped oscillators 371
 - in oviposition rhythm 53
 - in photoperiodism 340, 364, 368, 396, 404, 481
- Hourglass timer-Oscillator clock model 397, 398, 401
- Hyalophora cecropia* 49, 140, 259, 264, 277
- Hylophila prasinana* 287
- Hyphantria cunea* 49, 275, 303, 335
- Hypoxia 87
- Input pathway 107, 245, 260, 473
- Intensity thresholds 94, 95, 331, 334
- Interactions between parasites and hosts 319, 320
- Internal coincidence model 397, 401, 402, 405
- Internal Zeitgeber 39
- Isoinduction surface, see also Circadian Topography
- Jadera aeola* 276
- Juvenile hormone 120, 123, 156, 157, 158, 172, 280, 283, 292
- Kaniska canace no-japonicum* 287
- Laemostenus* spp. 13
- Lampyrus noctiluca* 178, 180
- lark 105, 108, 478
- Larval 'jumping' rhythms 55
- Larval wandering 56
- Larval diapause 277, 279
- Larval ecdyses 138, 266
- Lasius niger* 453
- Laspeyresia*(=*Cydia*) *pomonella* 279, 321, 323
- Lateral neurons 258
- Latitude 96, 322, 388
- Leptinotarsa decemlineata* 275, 283, 297, 301, 302, 332, 333, 334, 434
- Leucoma salicis* 302, 307, 327, 330
- Leucophaea maderae*
- Aschoff's rule 14, 15, 16
 - bimodality 11
 - constant light 10, 14
 - cuticle formation 15
 - D₂O effects 20
 - developmental plasticity 25, 26
 - entrainment 28, 29, 30
 - free-running rhythm 9, 10
 - lithium effects 20
 - location of clock 260
 - locomotor activity 9, 10
 - nymphal activity 12
 - optic lobes 198, 260
 - phase response curve 31, 32, 33
 - photoreceptors 247
 - red light effects 35
 - splitting 192
 - temperature compensation 17
 - temperature cycles 36
- Leudorphia japonica* 277
- Life cycles 120
- Light compass reaction 453
- Light growth effect 292
- Light intensity 94, 331
- Light responses 225
- Limenitis archippus* 293
- Limit cycles 218, 220
- Lithium, effects of 20
- Location of clock 245, 444

- Locomotor activity 8, 245
Locusta migratoria 150, 164, 283, 296, 321
 Long day responses 300
 'Long range' timer 292, 449, 470
 Longevity 208
Loxostege sticticalis 318
Lucilia caesar 275, 277, 312
Lucilia cuprina 16, 17, 33, 51, 56, 64, 69, 81, 100, 205
Lucilia sericata 312
 Luminescence 178
 Lunar rhythms 449, 463, 481
Lycaena phloea 287, 289
Lygaeus kalmii 295
Lymantria dispar 165, 167, 170, 285, 328
Lyperosia irritans 275, 312
 Malpighian tubules 118, 191
Mamestra brassicae 169, 278, 303, 323, 346, 353, 362, 379, 387
Manduca sexta 57, 124, 140, 143, 267, 278, 382, 439, 440, 445
 Masking 23, 27
 Maternal induction of diapause 281, 311
 Mating, rhythms of 184
Megachile rotundata 93
Megoura viciae
 action spectra 331, 333
 Bistability experiments 363, 367
 Bünsow experiments 345, 346, 367
 critical day length 300, 316
 egg diapause 275
 hour glass 364, 396, 423
 Nanda-Hamner protocol 353, 358, 364
 night interruption 342, 365, 366
 photoperiodic counter 387
 photoperiodic response 291, 300
 photoreceptors 434, 436, 437, 443
 seasonal morphs 291
 sensitive stage 312
 site of clock 437
 spectral sensitivity 332
 symmetrical skeleton photoperiods 363, 367
 transgeneration timer 470
 Veerman-Vaz Nunes protocol 368, 369
Melanoplus mexicanus 211
 Melatonin 137, 154, 155
 Metamorphosis 121
Metriocnemus knabi 310, 334, 342
Metrioptera hime 59
 Microilluminators 436, 437
 Midgut 154
 Migration 272, 294
Mimus tiliae 277
 Moulting 120
 Multioscillator circadian system 135, 139, 190, 401
Musca autumnalis 275
Musca domestica 21, 168, 194, 257, 296, 302, 475
 Mutants (see separate entries)
Myrmeleon obscurus 15, 464
Myzus persicae 291
 Nanda-Hamner protocol 351, 353
Nasonia vitripennis
 Bünsow protocol 345, 347
 circadian rhythms in photoperiodism 345-347, 352-355
 effect of chilling 316
 effect of diet 319, 389
 effect of host 319, 320
 effect of temperature 383, 385
 geographical strains 330, 331
 host deprivation 319
 internal coincidence 355, 405
 larval diapause 275
 maternal induction 311, 320
 Nanda-Hamner protocol 352, 353, 354, 355
 night interruption 342
 photoperiodic counter 379, 380, 381, 382, 385, 389
 photoperiodic response 300
 photoperiodic reversal 316
 resonance experiments (see Nanda-Hamner)
 spectral sensitivity 332, 334
 thermoperiod 308, 407
 transfer to DD 405, 406
Nauphoeta cinerea 10, 14, 31, 193
Nebria brevicollis 303
 Neck ligation 125
 Negative feedback 103, 222
Nemobius yezoensis 289
Neoconocephalus triops 296
Nephotettix apicalis 290
Nephotettix cincticeps 290
 Neural architecture 260
 Neurosecretory cells 123, 124, 292
 Night interruption experiments 341
 Night temperature 307
 Nitrogen anaesthesia 88
no-receptor-potential-A 108
Nomadacris septemfasciata 276, 305, 327
 Non-parametric entrainment 62
 Non-photoc Zeitgeber 39
 Ocelli 247-249, 253-254, 256, 259, 263, 434
Oedipoda miniata 284, 327
 Olfactory response 176
 Oligopause 274
Oncopeltus fasciatus 60, 150, 184, 295, 296

- Oogenesis 162
 Oostatic hormone 163
 Opsins 443
 Optic lobes 252, 260
 Optic lobe transplants 252
Orgyia antiqua 284
Orgyia thyellina 288
Oryctes rhinoceros 150
 Oscillator models 214
 single 217
 population models 228, 232
 Oscillators
 in cells and tissues 190, 191
 in photoperiodism 339
Ostrinia nubilalis
 bistability 78
 Bünsow experiments 346
 dual system theory 402
 effect of temperature 316, 317
 geographical strains 325
 hour-glass 365
 isoinduction surface 350, 351
 larval diapause 275, 277, 279
 light and dark periods 350, 351
 Nanda-Hamner protocol 353, 365, 370
 neurosecretory cells 123
 night interruption 342
 oviposition rhythm 51, 52, 64, 74, 78
 phase response curve 64
 phase relationship 74
 pheromones 174
 photoperiodic counter 378
 photoperiodic response 300, 301
 range of entrainment 74
 sensitive stage 310, 311
 thermoperiod 307, 309
 Output pathway 108, 245, 260, 478
 Ovary 109, 163
 Ovarian development 162
 Ovarian diapause 283
 Overt rhythms 414, 425
 Oviposition rhythms 51, 52, 185
 Ovulation and egg transport 163
 Oxygen tension 87

 Pacemakers, see also A-oscillators 66, 68, 119, 200, 246, 249, 254, 473, 478
Pachymorpha sexguttata 256
Panonychus ulmi 307, 318, 332, 334, 342
Panorpa vulgaris 389
Panstrongylus megistus 53
Papilio xuthus 288
 Parabiosis 249
 Parapause 274
 Parametric entrainment 62
Parasarcophaga similis 320

 Parasitoids 319, 320
Paravespula vulgaris 151
Parcoblatta pensylvanicus 297, 360
 PAS domain 105,
Passalus cornutus 13
Patrobis atrorufus 303
Pectinophora gossypiella
 abnormal light/dark cycles 352
 asymmetrical skeletons 79
 bistability phenomenon 361, 362, 363
 effect of diet 318
 egg hatch rhythm 44, 53, 54, 94, 153, 205
 embryonic development 293
 external coincidence model 414, 415
 geographical strains 326
 larval diapause 275
 multioscillator system 205
 Nanda-Hamner protocol 352
 night interruption 342
 oviposition rhythm 44, 51, 52, 60, 205
 photoperiodic response 300, 301, 414
 pupal eclosion 205, 425
 resonance experiment 352
 selection for 'early' and 'late' 99, 417
 selection for diapause 328
 sensitive stage 310
 spectral sensitivity 94, 153, 332, 333
 sperm movement 165
 temperature cycles 307
 T-experiment 359, 415, 416
Pegomyia hyosciami 316
period (per, PER) 10, 103, 104, 117, 127, 153, 154, 157, 167, 194, 195, 256, 259, 262, 283, 426, 427, 471, 477, 479

Periplaneta americana
 Aschoff's rule 15
 daily growth layers 151
 entrainment 28
 location of clock 249
 location of photoreceptors 247
 locomotor activity 8, 9, 10
 lunar rhythm 463
 PTTH release 126
 spectral sensitivity 35, 36
 Peripheral oscillators 117
Peripsocus quadrifasciatus 323
 Phase relationship 28, 33, 47, 481
 Phase response curve 30, 31, 38, 63, 64, 73, 131, 206, 218, 226, 262, 348, 419
 Phase shifts 30
 Pheromones 159, 168
 Pheromone biosynthesis-activating neuropeptide (PBAN) 159, 172, 173-176

- Philonthus fuscipennis* 305
Phormia terraenovae 209, 260, 320
 Phosphorylation 111
Photinus spp. 179, 180, 181
Photuris spp. 178, 179, 180
 Photoinducible phase 344, 397, 405, 419, 427, 428
 Photoperiodic clock 300
 location 444
 models for 396, 397
 Photoperiodic counter 368, 377, 383, 392, 393, 396
 Photoperiodic induction 271, 395, 396, 473, 479
 Photoperiodic photoreceptors 434
 Photoperiodic response 299, 313
 curves 300
 genetics 327
 modifications 313
 Photoperiodic reversal 341
 Photoperiodic termination 162, 163
 Photoreceptors 246, 247, 434, 439, 440
 Phototransduction cascade 443
Pieris brassicae
 Bünsow experiments 346
 effect of temperature 300, 314, 316
 genetics 330
 geographical strains 322, 330
 Nanda-Hamner protocol 352, 355, 358
 night interruption 342, 427
 photoperiodic receptors 439, 443
 photoperiodic response 300, 301
 pupal diapause 278
 resonance experiments 346, 352, 353, 358
 spectral sensitivity 332, 334
 symmetrical skeletons 361
 thermoperiodic induction 307, 308, 407
Pieris napi 287, 326, 327
Pieris occidentalis 287
Pieris rapae 277, 326, 342, 350, 427
pigment dispersing factor (pdf) 105, 108, 260, 262
Pimpla instigator 332, 352
Pioneer forficatis 328
 Planetary movements 1
Platynota spp. 170, 309
Plautia stali 441
Plodia interpunctella 276, 309, 346, 348, 352, 365
Plutella maculipennis 292
Polistes chinensis 297
Polychrosis botrana 311
Polygonia c-aureum 287
Polypedium vanderplanki 273
Povilla adusta 464
Precis coenia 289
 Primary range of entrainment 33
 Princeton model 218
 Programming of CNS 392
 Prothoracic glands 109, 123, 132, 133, 277, 278, 281
Protophormia terraenovae 13, 33, 441, 445
Pseudaletia unipuncta 170
Pseudosarcophaga affinis 328
Psylla pyri 290
Pteronemobius spp. 289, 328, 352, 441
Pterostichus nigrita 305, 352, 358, 370, 441
Pterostichus spp. 305
 PTTH 120, 123, 124, 125, 126, 134, 136, 156, 204, 278, 279, 281, 282, 479
 Pupal diapause 277
 Pupal eclosion 44, 262
 Pupation rhythms 57
Pyrrhocoris apterus 275, 283, 284, 294, 303, 351, 387, 444
 Quantitative models 216, 397, 403
 Quantitative responses to photoperiod 304
 Quiescence 273
 Range of entrainment 33, 73
 Rate of development 210
 Recovery from heat stress 296
 Reproductive diapause 161, 283
 Reproductive physiology 161-163
 Required day number 378-391
 Resonance (see Nanda-Hamner) 351, 397, 398
Rhagium inquisitor 13, 93
Rhodnius prolixus
 brain clock 134
 dorsal neurons 128
 ecdysis 139
 ecdysteroids 131, 132
 melatonin 137
 multioscillator clock 135, 136
 oviposition rhythm 51, 53
 PER cycling 134
 prothoracic glands 132, 133, 136
 PTTH release 126, 127, 128
 Rhodopsin 107
 Rhythm shattering 230, 231
 Ring gland 109, 281, 282
Riptortus clavatus 441
Romalea microptera 255
 Rubidium 21
 S-antigen 258
Samia cynthia 57, 58, 125, 130, 142, 259, 267
Sarcophaga spp., diapause 277, 281
Sarcophaga argyrostoma
 abnormal light/dark cycles 83, 350
 as host pupa 319
 asymmetrical skeleton photoperiods 79, 422, 424, 425

- bistability phenomenon 78, 362, 420, 421
 Bünsow experiments 346, 347
 constant light 82
 circadian rhythms in photoperiodism 339
 damping action of light 81
 eclosion rhythm 49, 51, 82, 83
 external coincidence model 418
 effect of temperature 314, 315, 384
 growth rate 293, 294
 larval 'wandering' 56
 multioscillators 205
 Nanda-Hamner protocol 353, 355, 356, 357, 370, 371
 night interruption 342, 366
 phase response curves 64, 66, 69, 70, 348
 photoperiodic counter 380, 381, 382, 384, 385, 387, 388, 390, 392
 photoperiodic response 301
 pupal diapause 275, 281
 pupal eclosion 49
 required day number 382, 384
 resonance (see Nanda-Hamner)
 sensitive period 391
 singularity 86
 spectral sensitivity 332
 symmetrical skeletons 361
 T-experiment 359, 360, 422
 temperature 317, 393
 thermoperiod 408
 transfer to DD 405, 406
 transformation curve 75
 ultrashort photoperiods 409, 410, 411
 Veerman-Vaz Nunes protocol 374
Sarcophaga bullata 286, 313, 328
Sarcophaga crassipalpis 281, 282, 286, 313, 427
Sarcophaga ruficornis 276
 Scalloping 237
Schistocerca gregaria 27, 59, 150
Scopeuma stercoraria 44
 Seasonal morphs 272, 286
 Selection experiments 99
 for diapause response 328
 for 'early' and 'late' eclosion 417
Semiadalia undecimnotata 295
 Semilunar rhythms 449, 455, 481
 Sensitive period 310
 Sequential changes in photoperiod 304
 Serotonin 39
Sesamia nonagrioides 169
 Sexual behaviour 168, 184
 Short day responses 302
Simulium spp. 14
sine oculis 263
 Singularity 32, 84, 92, 194, 218, 219
 Skeleton photoperiods 74, 76, 360, 420
 Slaves, see also B-oscillator 200, 473, 478
 Social Zeitgeber 39, 474
 South Pole 2
 Spectral sensitivity 35, 94, 331
 Sperm movement 164
 Spermatogenesis 163
Spilarctia imparilis 280
Spilosoma menthastris 307, 330
 'Splitting' 191, 229
Spodoptera littoralis 165, 169
 Stationary photoperiods 300
Stenocranus minutus 290
Stenotarsus rotundatus 276
 Stridulation 182, 296
 Subjective day and night 31, 63
 Suboesophageal ganglion 285
 Summer diapause 275
 Summer morph producing hormone (SMPH) 289
 Sun orientation 449, 453
Supella longipalpa 177
 Survival 208
 Symmetrical skeleton photoperiods 360
 Synthesis-Loss model 215

Tachinus sp. 305
Tachyporus sp. 305
take out 105, 108, 157
Teleogryllus commodus 11, 16, 35, 183, 194, 196, 204, 253, 254, 260
 Temperature coefficients 18, 46, 215, 227
 Temperature compensation 3, 17, 19, 45, 111, 462, 473, 476
 Temperature cycles 38, 88, 93
 Temperature effects on photoperiodism 313, 383
 Temperature-sensitive steps between photoreceptor and clock 92
 Temperature responses 226
 Temperature steps 18
Tenebrio molitor 14, 16
 Testis rhythms 164-166
Tetragoneura cynosura 275
Tetranychus urticae
 Bistability 363
 Bünsow experiments 346
 circannual rhythm 469
 counter 382, 389
 Hourglass timer-Oscillator counter model 398
 latitude 324
 Nanda-Hamner protocol 352, 357, 358, 370
 night interruptions 346
 photoreceptors 442
 resonance experiments 346, 352, 358
 Veerman-Vaz Nunes protocol 368
 vitamin A 442
 T-experiments 358, 415, 422
 Threonine-Glycine repeats 111

- Thalassomyia frauenfeldi* 460
Thalassotrechus barbarae 464
Therioaphis maculata 211
Thermobia domestica 164
 Thermoperiod 307, 407, 442
 Threshold 224
Thyanta calceata 290
Thymelicus lineola 285
 Tidal rhythms 449, 464, 481
 Time-compensated sun orientation 449, 453
timeless 104, 105, 108, 112, 127, 157, 480
 Token stimuli 273
 Transformation curve 72
 Transgeneration timer 470
 Transients 30, 47
 Translocation experiment 451
Triatoma infestans 53
Triatoma phyllosoma 53
Trichoplusia ni 170, 176, 259
Trogoderma glabrum 210
 Troglobites 13
 Troglophiles 13
 Troglloxenes 13
 Tropical diapause 275
 Twilight 474

 Ultradian rhythms 27, 195, 449, 471, 481
 Ultrashort photoperiods 409, 410, 411

 Van der Pol oscillators 219
 Veerman-Vaz Nunes protocol 368
Velia currens 14, 455, 456
 Vitamin A 442
 Vitellogenesis
 photoperiodic control 283
 rhythm of 162
vrille 105

 Winter coloration 272
Wyeomyia smithii 275, 323, 328

 X-Y oscillators 235, 236, 238

Yponomeuta vigintipunctatus 301

Zaprionus spp. 52
Zeitgedächtnis 4, 449, 450, 452
Zeitgeber 2, 3, 28, 62, 473, 474, 482
Zeitnehmer 108, 475